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RELATION BETWEEN QUINOID STRUCTURE AND BACTERIO- STATIC ACTIVITY OF TETRAMETHYL-DIAMINODI- PHENYLMETHANE DERIVATIVES

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Received for publication August 1, 1945

Bacteriostatic assays conducted with certain triphenylmethane dyes and their leuco derivatives have been published in a foregoing paper (Fischer, Hoffmann, Prado, and Boné, 1944). Among other results, it was found that the dye salt, the carbinol base, the bisulfite, and hydrosulfite derivatives of malachite green are bacteriostatically active against a strain of scarlet fever streptococcus (Dochez, N. Y. 5), all of them practically at the same level, whereas the leuco base, the leucocyanide, and the leucoamine have no action of comparable extent.

It is somewhat difficult to draw definite conclusions from these facts about the relations between the chemical structure and the bacteriostatic activity of the substances in question. On first sight, no common features can be found among the members of the active group. The dye salt possesses a very particular structure, different from that of any colorless derivative. We cannot discuss here the real nature of the dye structure, which has been the subject of different theories. We adopt here simply the quinoid theory and shall use the expression "quinoid" and the corresponding chemical formulas to designate that particular structure on which the dye character depends, whatever the real nature of that structure may be.

Besides the dye salt, three other substances, the carbinol base and the hydrosulfite and bisulfite derivatives, also showed bacteriostatic activity, as already mentioned. These substances are leuco derivatives and lack in consequence a quinoid structure. They are, however, able to obtain such a structure with relative facility. The easy transformation into the dye salt by the action of certain acids represents a most characteristic chemical property of the carbinol bases. The same can be stated for the bisulfite and hydrosulfite derivatives which behave like "vat dyes," that is, transform into dye substance in the air if moistened, forming dye carbonate (Wieland, 1919). Thus, an actually present or easily achieved quinoid structure may be looked upon as a possible common feature of the bacteriostatically active derivatives.

In contrast to these, the leuco base which is inactive must be oxidized before it can change into a quinoid substance. Other relatively inactive derivatives, that is the leucocyanide and the leucoamine, although not requiring an oxidative process for their quinoid transformation, are considerably stabler compounds than the members of the active group and resist to a greater extent the action of acids.

It is possible to assume as a working hypothesis that the bacteriostatic activity of malachite green dye and its derivatives is connected with their quinoid structure and that the efficacy of leuco derivatives depends entirely on their greater or lesser ability to take up such a structure under different circumstances.

It is true that during our former experiments no appearance of color was observed to indicate the formation of dye salts in the culture medium. This does not exclude, however, the possibility of a local transformation inside the bacterial cell. We have again tried to approach the problem by repeating our experiments with certain diphenylmethane homologs of the triphenylmethane derivatives tested before. The diphenylmethane derivatives offer the advantage that their leuco compounds are rather stable substances, having in general no "vat dye" properties (Albrecht, 1894; Weil, 1894). The structure of the diphenylmethane series admits, furthermore, the formation of more numerous derivatives, which circumstance enables the realization of completer and more varied comparative assays.

Among the diphenylmethane dyes, auramine has long been used as an anti-septic (*pyocyaninum aureum*). Its bacteriostatic effect is, according to Kligler (1918), considerably weaker than that of triphenylmethane dyes. Contrasting results have been obtained by Fairbrother and Renshaw (1922) in bactericidal tests in which auramine showed a very marked efficacy.

EXPERIMENTAL

Our experiments have been performed with a technique very similar to that described in the foregoing paper. We used in the recent assays a *Staphylococcus aureus* strain instead of the scarlet fever streptococcus strain, but we also checked the most important results with the latter organism.

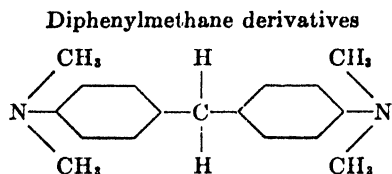
The substances to be tested were dissolved uniformly in 70 per cent acid alcohol in a concentration of 1 per cent. The desired dilutions were made by mixing the stock solution with the necessary quantity of peptone broth.

Dye salt of Michler's hydrol, the corresponding leuco base, and Michler's ketone. Michler's hydrol, or tetramethyl-diaminodiphenylmethanol, forms with acids deep blue salts which are diphenylmethane homologs of malachite green salts. Michler's hydrol itself is, correspondingly, the homolog of the carbinol base. Tetramethyl-diaminodiphenylmethane can be conceived as its leuco base, analogous to leucomalachite green. Michler's ketone has no triphenylmethane homolog. The structures of these compounds are compared in figure 1.

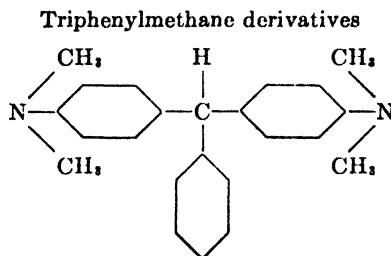
We tested these compounds in bacteriostatic experiments. Michler's hydrol was not included because it readily transforms with acids into the dye salt. In fact, the dye salt solution has been made by dissolving Michler's hydrol in acid alcohol (70 per cent). The results of the bacteriostatic tests are given in table 1.

Table 1 demonstrates: (1) The diphenylmethane homolog of malachite green, that is, the dye salt of Michler's hydrol, has a considerably weaker bacteriostatic effect than malachite green. It is clear that the removal of the benzene ring diminished the efficacy (Kligler, 1918). (2) Among the diphenylmethane

derivatives tested, only the quinoid dye salt showed bacteriostatic action, whereas the leuco derivatives were inactive.

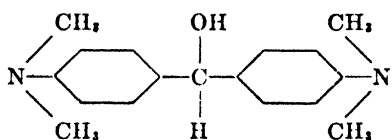


Tetramethyl-diaminodiphenylmethane

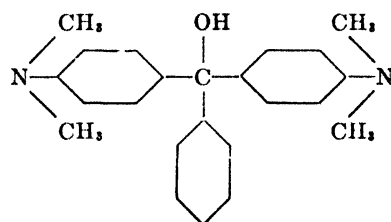


Leucomalachite green

LEUCO BASES

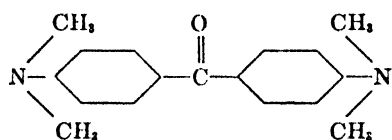


Tetramethyl-diaminodiphenylmethanol
(Michler's hydrol)



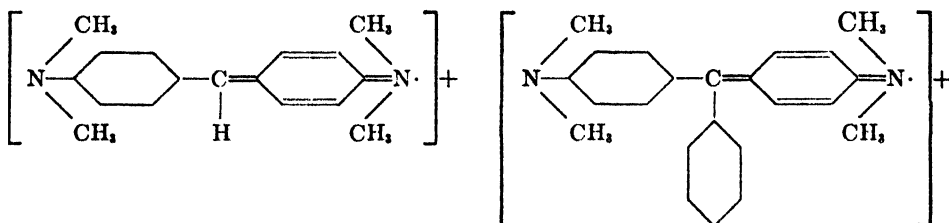
Malachite green carbinol

CARBINOL BASES



Michler's ketone

KETONE



Dye cation of Michler's hydrol

Malachite green cation

DYE CATIONS

FIG. 1

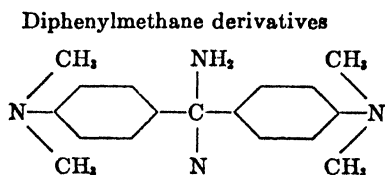
Amine and imine derivatives of tetramethyl-diaminodiphenylmethane. The triphenylmethane structure of malachite green permits only one amine derivative,

the leucoamine. In the corresponding diphenylmethane series, there are two amine and one imine derivatives. Among them, only one, auramine, has a quinoid dye structure. The imine derivative has the same relation to the aura-

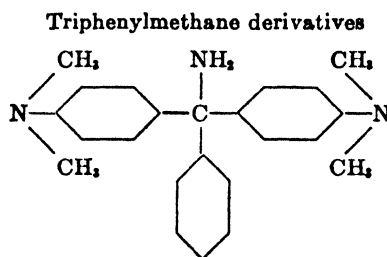
TABLE 1
Bacteriostatic action against Staphylococcus aureus

	DILUTIONS OF THE SUBSTANCES TESTED				
	1/5,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
Malachite green dye	—	—	—	—	+
Dye salt of Michler's hydrol.	—	—	+	+	+
Tetramethyl-diaminodiphenylmethane .	+	+	+	+	+
Michler's ketone.	+	+	+	+	+

— = no growth after 24 hours; + = regular growth after 24 hours.

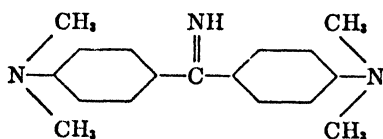


Leucoauramine



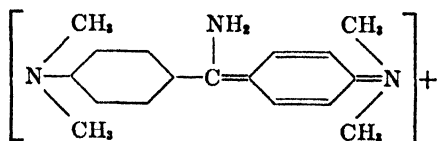
Leucoamine of malachite green

LEUCO BASES



Auramine base

IMINE BASE



Auramine cation

DYE CATION

FIG. 2

mine salts as carbinol bases have to their dye salts. It is therefore called "auramine base." The third compound, the leuco base of auramine, is the proper homolog of the leucoamine of malachite green. The structure of these substances is given in figure 2.

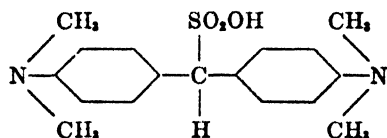
Bacteriostatic tests have been realized only with auramine and its leuco base, because the auramine base transforms with acid into auramine dye salts. The results are summarized in table 2.

Table 2 shows: (1) The quinoid-structured amine derivative, i.e., the auramine dye salt, is bacteriostatically active, whereas its leuco base, without such a structure but otherwise almost entirely of the same chemical composition, is without activity. (2) The bacteriostatic activity of the auramine dye salt is of the same order of magnitude as that of the dye salt of Michler's hydrol, in spite of the fact that the two differ from each other by an amine group. These facts

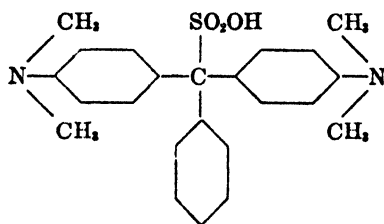
TABLE 2
Bacteriostatic action against Staphylococcus aureus

	DILUTIONS OF THE SUBSTANCES TESTED		
	1/5,000	1/10,000	1/100,000
Dye salt of Michler's hydrol	—	—	+
Auramine	—	—	+
Leucoauramine	+	+	+

— = no growth after 24 hours; + = regular growth after 24 hours.



Tetramethyl-diaminodiphenylmethane
sulfonic acid



Tetramethyl-diaminotriphenylmethane
sulfonic acid
(Bisulfite derivative of malachite green)

FIG. 3

clearly demonstrate the prime importance of the quinoid structure in relation to the bacteriostatic activity among the derivatives of tetramethyl-diaminodiphenylmethane.

Methane sulfonic derivative. This compound is the homolog of the bisulfite derivative of malachite green. From our point of view, however, there is a very significant difference between these substances—the bisulfite derivative of malachite green being a “vat dye” (Wieland, 1919), whereas the diphenylmethane sulfonic derivative is a rather stable compound (Weil, 1894). Figure 3 shows the formulas and table 3 the bacteriostatic activities of these substances.

Table 3 shows that the stable diphenylmethane derivative without quinoid structure is inactive, whereas the triphenylmethane derivative with “vat dye” character has an activity corresponding to that of malachite green dyes.

Nitric, carboxamide, and carboxylic derivatives of auramine and Michler's hydrol. The formulas of these compounds are shown in figure 4.

The amino series shows no quinoid structure. The members of the hydroxy series can form quinoid salts with acids, but these are hydrolyzed by water and exist practically only in solid form (Albrecht, 1894). Correspondingly, their solutions in acid alcohol (70 per cent) had no dyeing properties and showed only a weak brownish-green color. In the bacteriostatic test all these compounds were inactive to a concentration of 1:5,000.

TABLE 3
Bacteriostatic action against Staphylococcus aureus

	DILUTIONS OF THE SUBSTANCES TESTED				
	1/5,000	1/10,000	1/100,000	1/1,000,000	1/10,100,000
Tetramethyl-diaminodiphenylmethane sulfonic acid	+	+	+	+	+
Tetramethyl-diaminotriphenylmethane sulfonic acid	—	—	—	—	—

— = no growth after 24 hours; + = regular growth after 24 hours.

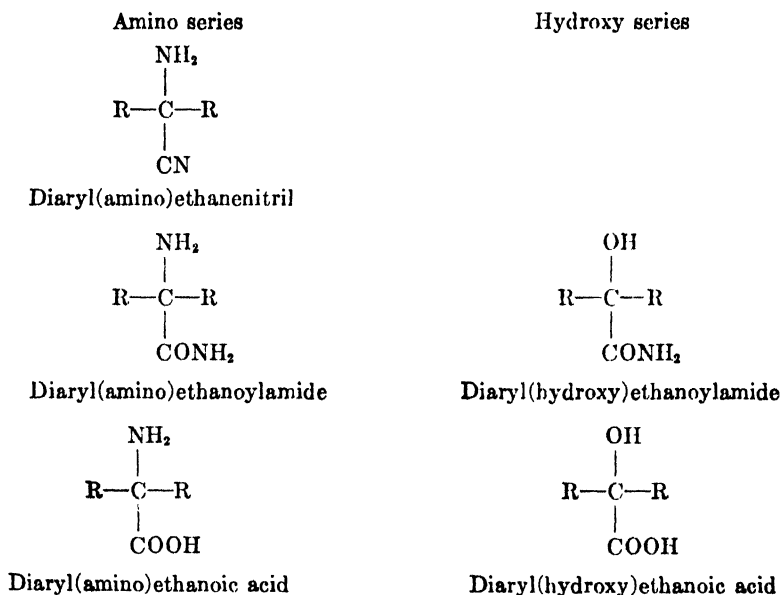


FIG. 4

Assays with scarlet fever streptococcus. As mentioned, the most important compounds have also been tested on the scarlet fever streptococcus strain used in the previous experiments (Fischer *et al.*, 1944). Table 4 shows that the results are in complete accord with those obtained in experiments on *Staphylococcus aureus*.

Preparation of the substances used. *Auramine chloride:* An aqueous solution of commercial suramine was precipitated by diluted ammonia. The base obtained

was redissolved with a slight excess of HCl in 70 per cent alcohol. *Michler's ketone*: A concentrated auramine solution was boiled for three hours with an excess of HCl. The ketone was then precipitated by cooling and recrystallized from hot alcohol. Melting point: 163 C (reported, 172 C). *Michler's hydrol and leucoauramine*: These compounds have been prepared by the reduction of Michler's ketone and of auramine, respectively, and recrystallization. Melting points: Michler's hydrol, 96 C (theoretical, 96 C); leucoauramine, 135 C (theoretical, 135 C). *Methane sulfonic acid* was prepared according to Weil (1894), and the *aminoethanenitril, amino- and hydroxy-ethanoylamide, and ethanoic derivatives* were made according to Albrecht (1894). Melting points: ethanenitril, 125 C (Albrecht, 130 C); aminoethanoic acid, 171 C (Albrecht, 171 C); hydroxy-ethanoylamide, 164 C (Albrecht, 162 to 163 C). *Tetramethyl-diaminodiphenylmethane* was prepared by condensing 16 parts of dimethylaniline with 6 parts of formaldehyde in the presence of 20 parts of a 25 per cent solution of HCl. After boiling some hours, the methane derivative was precipitated by ammonia solu-

TABLE 4
Bacteriostatic action against Streptococcus pyogenes

	DILUTIONS OF THE SUBSTANCES TESTED			
	1/10,000	1/100,000	1/1,000,000	1/10,000,000
Auramine	—	+	+	+
Michler's hydrol .	—	+	+	+
Michler's ketone	+	+	+	+
Auramine carboxamide	+	+	+	+
Malachite green	—	—	—	+

— = no growth after 24 hours; + = regular growth after 24 hours.

tion, recrystallized from hot alcohol, dissolved in diluted HCl, precipitated by ammonia solution, and finally recrystallized from benzene. Melting point: 84 C (reported, 90 to 91 C).

DISCUSSION

The results of our experiments with different derivatives of tetramethyl-diaminodiphenylmethane allow the conclusion that the bacteriostatic action depends, in this series, only on the quinoid structure. Substances with otherwise relatively similar structures behaved in a basically different manner in bacteriostatic experiments if they differed with respect to the presence or absence of quinoid structure. On the other hand, substances relatively more different from one another but with the same quinoid structure showed a practically equal bacteriostatic activity.

There are some facts which support the hypothesis of the existence of a similar relation in the triphenylmethane series, but the lability of the corresponding leuco derivatives does not allow a clear demonstration of this relationship.

SUMMARY

Among different derivatives of tetramethyl-diaminodiphenylmethane only those with a quinoid dye structure, that is, the dye salts of Michler's hydrol and of auramine, have bacteriostatic activity.

A relatively slight change in the composition of these substances is sufficient to deprive them of their bacteriostatic efficacy if this change causes the loss of the quinoid structure.

The introduction of an amine group in the central methyl radical does not alter the bacteriostatic activity if the quinoid structure remains intact.

The two active tetramethyl-diaminodiphenylmethane derivatives are considerably less active than malachite green dye. It must, therefore, be assumed that the removal of a benzene ring is apt to diminish the efficacy.

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VARIATION IN *PENICILLIUM NOTATUM* INDUCED BY THE BOMBARDMENT OF SPORES WITH NEUTRONS¹

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Investigators who have studied the production of penicillin by *Penicillium notatum* are well aware of the fact that individual strains of this mold differ greatly in their capacity to produce the antibiotic substance. Since it is reasonable to suppose that the reactions involved in the formation of antibiotic substances may be genetically controlled biochemical reactions, it seemed probable that induced variation of *P. notatum* might bring forth more highly productive strains.

In the attempt to induce variation of fungi, many workers have employed such agents as heat, chemicals, ultraviolet light, and x-rays. Although positive results on mutations produced by chemical treatment have been reported, it is questionable whether the substances used could have reached the nuclei of the organisms so as to bring about demonstrable genetic changes. Since the discovery that x-rays induce gene mutations, radiation has become an important tool in genetic research, and in all organisms adequately tested the so-called ionizing radiations have been found to produce mutations (Fano and Demerec, 1944).

Barnes (1928) isolated variant forms arising from heated spores of *Eurotium herbariorum*, and Christensen (1929), working with *Helminthosporium sativum*, reported that some mutants obtained after cultures had been exposed to high temperatures remained stable for at least four years although some reversions did occur. Dickson (1932) obtained saltations after he had exposed both mycelium and spores of *Chaetomium cochliodes* and other fungi to ultraviolet light and x-rays. Greaney and Machacek (1933) produced a white fertile saltation of *Helminthosporium sativum* by means of ultraviolet irradiation. The dermatophyte *Trichophyton mentagrophytes* was used by Hollaender and Emmons (1941) for study of the wave length dependence of mutation production in ultraviolet light.

Tatum and Beadle (1942) induced mutation of *Neurospora crassa* by x-ray treatment of perithecia and conidia. Genetic analysis of the mutants tested indicates that they differ from the parent by single genes. Tatum (1944) reported that the biochemical activities of *N. crassa* are genetically controlled and that there is no inherent reason why the control of analogous reactions should be fundamentally different in other fungi, such as *Aspergillus* and *Penicillium*, in which genetic analysis has not yet been accomplished.

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Marked variation appeared in cultures of *P. notatum* grown from spores which were bombarded with neutrons, and strains have been obtained which differ greatly in their ability to produce penicillin (Myers and Hanson, 1945). The present report concerns the different types of treatment to which this mold was exposed and the isolation, description, and testing of some variants produced.

PARENT CULTURE

All of the variants have originated from a strain designated as *Penicillium notatum* no. 17, which was isolated in December, 1943, after spores of strain NRRL 1249.B21² had been subjected to heat treatment. Up to the present time strain 17 has retained its vigor as a good producer of penicillin, and its appearance in culture has remained essentially constant. Moderately heavy spore suspensions in normal saline, moist spores, dry spores, and actively growing cultures were subjected to bombardment with neutrons.

TECHNIQUES INVOLVING NEUTRON BOMBARDMENT

Neutrons from a 42-inch cyclotron in the Department of Physics were employed in these studies.³ The conditions under which the spores were exposed to bombardment are described below.

Bombardment by slow neutrons. Test tubes holding saline suspensions of spores and plates of actively growing cultures of strain 17 were placed within a few inches of the wall of the cyclotron in the region nearest to the point of the target within the machine. The neutrons passed through the saline or agar whenever various targets were bombarded with deuterons. Boxes of paraffin were packed around the tubes and plates to slow down the neutrons and increase the probability of "hits." Neutrons recoiling from the hydrogen nuclei of the paraffin passed through the suspensions and cultures. Under these circumstances, the neutrons responsible for the induced variation were in the main relatively slow and might be considered to be "thermal" neutrons.

The first variants isolated were obtained from spores present in suspensions which had been exposed to neutrons for 1, 2, 3.5, 4.5, 5, 6, and 7 hours from a beryllium target bombarded by 10 Mev deuterons. Other targets were subsequently used, and the cyclotron was in operation approximately 5 days per week. Later isolates came from cultures of spores which had been at the cyclotron for 30, 44, and 134 days.

The actively growing cultures of strain 17 were subjected to bombardment with slow neutrons for 3 hours. Petri dishes containing approximately 15 ml of malt agar (Difco) and of a sporulation medium⁴ were streaked with spores 48,

² Strain NRRL 1249.B21 was received from Dr. K. B. Raper of the Northern Regional Research Laboratory, Peoria, Illinois, where it was developed for the production of penicillin in surface culture.

³ We wish to express our appreciation to Dr. M. L. Pool of the Department of Physics and Mr. Richard A. Forgrave, Technical Assistant at the Cyclotron, for suggestions and help in the experiments involving the use of the cyclotron.

⁴ The formula for this medium was obtained from Dr. A. J. Moyer of the Northern Regional Research Laboratory, Peoria, Illinois.

36, 24, 12, and 0 hours prior to exposure. Duplicate plates of each culture were treated, and similar controls were untreated.

Bombardment by fast neutrons. Spores from a 6-day-old culture of strain 17 were sealed within sterile glass ampoules small enough to fit into a cup behind the lithium target in the vacuum chamber of the cyclotron. The target was bombarded by 10 Mev deuterons, and the resulting neutrons passing through the ampoules had energies up to 24 Mev.

Some of the ampoules were dry whereas others contained a small amount of moisture. The treatment with neutrons was as follows: 0 minutes—dry spores (control); 15 minutes—dry spores; 30 and 180 minutes—moist and dry spores.

ISOLATION OF VARIANTS

The spore suspensions which had been bombarded with slow neutrons were plated out on malt agar and sporulation medium by two methods: (1) by streaking a loopful of suspension over the surface of the medium (approximately 15 ml) in a petri dish and (2) by placing a given amount of diluted suspension on the surface of the medium and then tilting the plate so as to obtain a uniform distribution over the agar. Throughout the entire course of this investigation all mold cultures were incubated at 25 C.

The outer surface of the glass ampoules which had been exposed to fast neutrons was sterilized chemically, and the ampoules were crushed into test tubes containing 5 ml of sterile distilled water. The tubes were thoroughly shaken to insure suspension of the spores, and the suspensions were plated out by the methods mentioned above.

Variants were isolated at different times during an incubation period of about 10 days. Criteria for recognition and selection were the texture, color, degree of sporulation, and other superficial characteristics of the colonies. A binocular colony microscope was of considerable help in this work. The growth in many plates showed such a marked degree of variation that the colonies selected for picking were but a very small portion of the total number of types observed. Isolations were made in duplicate by touching the central portion of the colony with the sharpened tip of a sterile inoculating needle and then streaking the surface of a slope of sporulation medium with the spores adhering to the needle. When the primary cultures showed growth of more than one type, they were further cultured to accomplish isolation.

GENERAL STUDIES ON VARIANTS

The number of variant colonies per plate increased as the time in which the suspensions had been exposed to bombardment with slow neutrons was lengthened. Thus the greatest amount of variation appeared in cultures from spores which had been bombarded longest. This was probably due to the fact that the longer the suspensions were exposed to neutrons, the greater the number of neutrons which traversed the suspensions and also the greater the number of probable "hits." Certain definite types of variation were frequently observed. This seems to indicate that characteristic changes are repeatedly produced when specific regions within the spore are struck.

Up to the present time approximately 150 isolations have been made. These are characterized by differences in color, texture, rate and quantity of vegetative growth and of sporulation, color and amount of transpired fluid, and color of the reverse of colonies. The color of sporulation includes various shades of yellow, orange, tan, green, and blue. The variants have, in almost all instances, bred true throughout a number of successive transfers. There has been no evidence of reversion from any of the different colors back to the original blue-green.

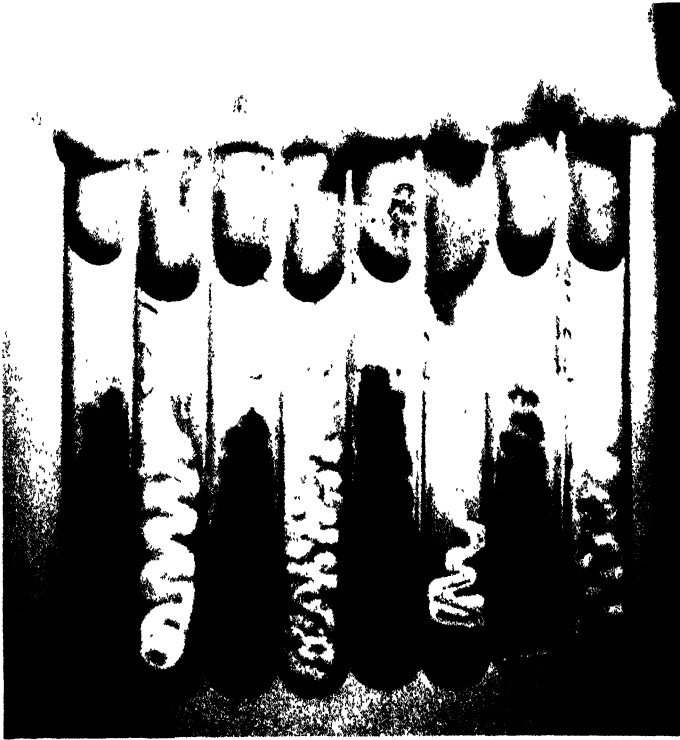


FIG. 1. FIVE-DAY OLD CULTURES ON SPORULATION MEDIUM OF SEVERAL TYPES OF VARIATION WHICH FREQUENTLY OCCURRED

The parent strain 17 is at the far left

Because such a wide variety of changes has occurred, all the variants cannot be sharply classified among a few types. Figure 1 shows several types ranging in color from white to blue-green which were frequently seen.

The actively growing cultures which were exposed to bombardment with slow neutrons were studied throughout an incubation period of several days. None of the cultures showed definite evidence of induced variation.

Two series of plates were inoculated with spores which had been treated with fast neutrons. Each plate in the first series was streaked with a loopful of suspension, and in the second series the surface of the medium in each plate was inoculated with 1 ml of suspension. The amount of growth and variation which was present in both series of plates at the end of 7 days' incubation is presented

in table 1. The variation which was observed was similar to that seen in cultures of spores which had been treated with slow neutrons. Several isolations were made from the 15-minute dry, and 30-minute moist, cultures. Among the isolates from the 15-minute dry was the buff-colored variant C3-6 which will later be described in detail.

TABLE 1

The relative amounts of growth and variation which occurred in two series of cultures from dry and moist spores that had been bombarded for various lengths of time with fast neutrons having energies up to 24 Mev

TIME ^a AND CONDITION OF EXPOSURE OF SPORES TO NEUTRONS	FIRST SERIES	SECOND SERIES
	Plates streaked with a loopful of spore suspension	Plates inoculated with 1 ml of spore suspension
0 minutes—dry	No variation	No variation
15 minutes—dry	Slight amount of variation	
30 minutes—dry	No growth	Slight growth
30 minutes—moist	No growth	Considerable growth and marked variation
180 minutes—dry	No growth	No growth
180 minutes—moist	No growth	No growth

STUDIES ON THE PRODUCTION OF PENICILLIN

The variants have shown marked differences in their ability to produce penicillin. A few no longer possess this property, whereas others apparently produce more than the parent strain. The capacity of the variant cultures to produce penicillin was studied first by a screening test for segregation of the better strains, and second by testing these more highly productive strains in surface culture. Although studies on the production of penicillin in submerged culture are incomplete, preliminary tests have shown that some of the variants give yields much higher than those of the parent—a strain originally developed for production in surface culture.

The medium and method used for the screening test and the method used for the production of penicillin in surface culture were those described by Raper, Alexander, and Coghill (1944) with the following exceptions: Throughout the course of this work dehydrated corn steeping liquor (approximately 94 per cent solids) was employed in amounts of 10 g per liter of the screening test medium and 50 g per liter of the medium for the production of penicillin in surface culture. All media were sterilized for 20 minutes at 15 pounds' steam pressure.

Screening test. All variants were subjected to the screening test. Strain 17 was included as a control along with each series of cultures tested. The screening test medium was distributed in 20-ml amounts in sterile, uniform petri dishes. In most cases duplicate plates were inoculated with spores of each strain tested. The plates were inverted and incubated for 6 days. On the sixth day, beginning at the edge of the colony, a radial series of 5 discs was cut with a cork borer

having an inside diameter of 5 mm. The discs were transferred to the surface of the medium seeded with *Staphylococcus aureus* FDA no. 209 in plates like those used in the cup assay for penicillin. The plates were incubated overnight at 37 C. The zones of inhibition were measured, and their diameters gave some

TABLE 2

Comparative diameters in millimeters of inhibition zones against Staphylococcus aureus as produced by a radial series of 5 agar discs cut from cultures of some variants isolated from suspensions receiving the longest bombardment

CULTURE	NUMBER OF DISC IN RADIAL SERIES				
	1	2	3	4	5
No. 17	27.5	24.0	19.5	14.0	8.0
C6-18	29.2	26.0	22.0	17.7	13.2
C6-19	30.2	26.7	23.0	18.2	13.5
C6-26	30.0	27.2	22.5	17.2	13.0
C6-28	30.0	27.2	23.0	17.5	12.5
C6-31	30.0	27.2	24.5	21.0	16.0
C6-32	29.2	26.0	22.2	19.2	14.5
C6-41	29.0	25.2	21.5	17.0	13.0
C6-49	31.0	27.5	23.7	19.5	14.7
C6-51	28.5	25.5	21.2	16.0	11.5
C6-52	29.7	26.0	21.5	16.5	13.0
C6-54	28.2	26.0	22.5	17.7	13.2

TABLE 3

Oxford units of penicillin per ml culture fluid and pH changes produced in surface culture by some variants isolated from suspensions receiving the longest bombardment

CULTURE	TIME IN DAYS							
	5		6		7		8	
	pH	Penicillin	pH	Penicillin	pH	Penicillin	pH	Penicillin
No. 17	6.5	102	7.2	140	7.7	148	7.9	164
C6-18	6.5	97	7.1	137	7.6	154	8.0	148
C6-19	6.4	90	7.0	147	7.5	178	7.8	164
C6-26	6.1	83	6.8	131	7.4	183	7.8	180
C6-28	6.5	102	7.6	137	7.7	160	7.9	164
C6-31	5.1	64	6.5	123	7.6	196	8.1	172
C6-32	6.7	104	7.1	149	7.7	162	8.0	145
C6-41	6.5	94	7.0	156	7.6	185	7.9	168
C6-49	4.6	15	6.1	54	7.5	103	7.8	126
C6-51	6.4	88	7.2	137	7.6	178	7.9	155
C6-52	6.5	97	7.2	156	7.6	183	8.0	148
C6-54	6.5	107	7.2	145	7.8	160	8.0	140

indication of the concentration of penicillin present at the sites from which the discs had been taken. The greatest amount of inhibition always occurred around discs taken adjacent to the colony, with the subsequent discs showing progressively less. Screening test results on some active producers which were isolated from suspensions receiving the longest bombardment are given in table 2.

The production of penicillin in surface culture. For the production of penicillin in surface culture 250-ml Erlenmeyer flasks containing 60 ml of medium were used. The medium in each flask was inoculated with 1 ml of a heavy suspension of spores. Strain 17 was included as a control along with each series of cultures tested. At daily intervals, generally from the fifth through the eighth day, samples of equal volumes were aseptically removed from all flasks. Like samples were pooled and used for pH determinations and assay for penicillin content. A glass electrode potentiometer was used for the pH determinations. The cylinder plate method as described by Schmidt and Moyer (1944) was used for all assays. The results obtained when quadruplicate cultures of some members of the C6 series were tested are shown in table 3.

Comparative Studies on a Buff-colored Variant (C3-6) and on Strain 17

The first buff-colored variant was isolated from a culture of spores which had been bombarded with fast neutrons and was designated as strain C3-6. Similar variation appeared rather frequently in cultures from the suspensions which had been treated longest. The color, morphology, and ability to produce penicillin of the parent no. 17 and of the variant C3-6 were studied and compared.

Color description of strains C3-6 and 17. The following observations were made from 7-day-old cultures growing on plates of sporulation medium: strain C3-6—vinaceous buff to avellaneous (pl. XL, Ridgway, 1912); strain 17—pea green to celandine green (pl. XLVII). Transpired droplets on colonies of C3-6 were a deep rose color whereas those on no. 17 were yellow. The reverse of colonies grown on the screening test medium also differed: C3-6 was a rosy color; no. 17 was a cream-yellow color.

Morphologic studies. Colonies of each mold seemed identical in all aspects except color and readily fit the descriptions of this species as given by Thom (1930) and Smith (1938). Microscopic studies were made of 48- and 72-hour slide cultures. The structure of the fruiting body was the same in the parent strain and the variant, and the average measurements of the hyphae, metulae, spores, and other portions showed the respective parts in strains C3-6 and 17 to be of virtually the same size.

Production of penicillin by strains C3-6 and 17. Eight flasks of medium for the production of penicillin were inoculated with spores of strain 17; a similar group was inoculated with spores of strain C3-6. At definite intervals throughout an incubation period of 10 days, samples were aseptically removed from all flasks. Like samples were pooled and used for pH determination and assay for penicillin content. The assay values and pH determinations are given in table 4 and graphically represented in figure 2.

Qualitative Studies on the Antibiotic Substance Produced by Sixty-Two Variant Strains

To determine whether there might be qualitative differences in the antibiotic substance produced, sixty-two of the variant strains were tested for activity against both penicillin-susceptible and penicillin-resistant species of bacteria. The test organisms used were strains of *Staphylococcus aureus*, *Bacillus subtilis*,

TABLE 4

Comparative changes in pH and the production of penicillin in surface culture by a buff-colored variant (C3-6) and strain 17

TIME	pH		PENICILLIN—OXFORD UNITS PER ML CULTURE FLUID	
	C3-6	No. 17	C3-6	No. 17
days				
4	5.0	5.3	26	41
5	6.4	6.5	77	81
6	6.6	6.7	121	122
6.5	6.9	7.0	129	129
7	7.3	7.3	165	170
7.5	7.5	7.6	177	179
8	7.8	7.9	170	178
9	8.2	8.1	136	142
10	8.2	8.1	112	122

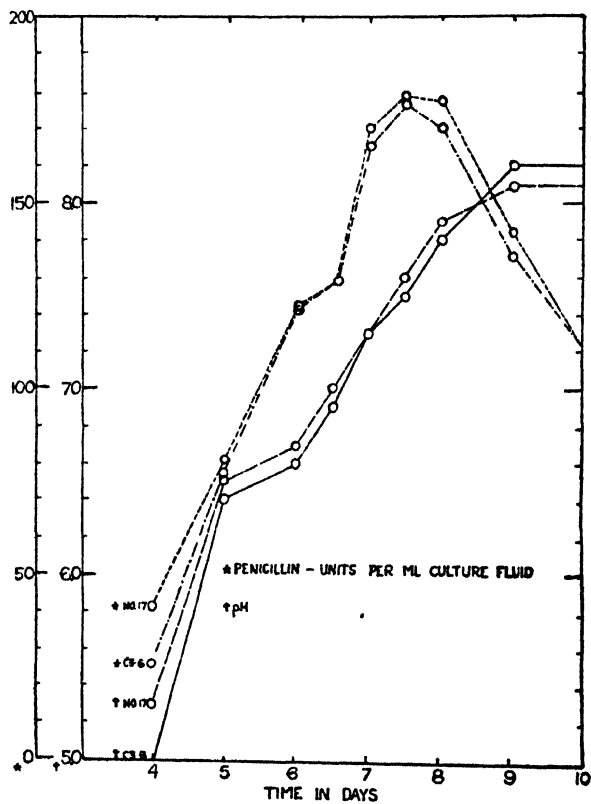


FIG. 2. COMPARATIVE CHANGES IN pH AND THE PRODUCTION OF PENICILLIN IN SURFACE CULTURE BY A BUFF-COLORED VARIANT (C3-6) AND STRAIN 17

Brucella abortus, *Shigella dysenteriae*, *Escherichia coli*, *Bacillus mycoides*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Proteus vulgaris*. The tests were made by cutting discs of agar adjacent to 6-day-old colonies grown on the screening test medium and placing them on pour plates of agar seeded with the respective test organisms. When judged by the presence of inhibition zones in the bacterial cultures, there appeared to be no qualitative difference in the inhibitory substance produced by those variants which were tested.

DISCUSSION

The experimental results presented in this report indicate that variant colonies arise from spores of *P. notatum* which have been bombarded with neutrons. The advent of the variation is probably a matter of chance, depending upon whether a definite region within a spore is struck in such a way as to produce an observable biological effect. It may well be that changes similar to those resulting from bombardment also occur spontaneously but at a much lower rate.

Whether the variants of *P. notatum* represent true gene mutations is not known since this species has revealed neither ascocarps nor zygotes, and thus genetic experiments involving studies of nuclear fusion and meiotic division cannot be performed. However, Tatum and Beadle (1942) and Tatum (1944) concluded from their studies on morphologically and biochemically mutant strains of *Neurospora crassa* produced by x-ray treatment of conidia and perithecia that the mutant character is inherited as if it were associated with a single gene mutation. They used heterokaryons made between different strains for tests of gene dominance in a manner similar to that used in dominance studies of the diploid cells of most plants and animals.

Continued study on variations induced by neutrons may lead to better understanding of the physiology and genetics of microorganisms such as bacteria, actinomycetes, fungi, and protozoa. The production of new strains may lead to finding new antibiotic substances as well as to obtaining strains capable of producing greater amounts of the known antibiotics.

SUMMARY

Marked variation appeared in cultures from spore suspensions of *Penicillium notatum* which were exposed to bombardment with slow neutrons for periods of time ranging from 1 hour through 134 days, and the number of variants increased as the time of exposure was lengthened.

Actively growing cultures which were treated with slow neutrons for 3 hours showed no definite evidence of induced variation.

Variation similar to that induced with slow neutrons occurred when moist and dry spores were bombarded with fast neutrons (energies up to 24 Mev) for 15 and 30 minutes; however, an exposure of 180 minutes was lethal to all spores.

Isolation and study were made of 150 variants which are characterized by differences in such aspects as color, texture, rate and quantity of vegetative

growth and of sporulation, color and amount of transpired fluid, and color of the reverse of the colonies.

A screening test, surface cultivation, and preliminary tests in submerged cultivation showed that the variant strains differ greatly in their capacity to produce penicillin, for some no longer possess this property whereas others give yields considerably higher than those obtained from the parent culture.

Comparative studies on the morphology and the production of penicillin in surface culture showed the parent strain and a buff-colored variant to be nearly identical except for color.

No qualitative differences were observed when the inhibitory substance produced by 62 variants was tested against 10 species of bacteria.

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A STUDY OF FLUID THIOLYCOLLATE MEDIUM FOR THE STERILITY TEST

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The use of sodium thioglycollate in a clear medium for the cultivation of anaerobic bacteria was introduced by Brewer in 1940. The following year the National Institute of Health adopted a liquid medium designated "fluid thioglycollate medium" for use in the testing of biological products for sterility. Two formulae were recommended. They differed only in that one (Brewer, 1941) specified beef infusion and 1.0 per cent of peptone, and the other (Linden, 1941) specified 2.0 per cent of peptone and 0.2 per cent of yeast extract but no meat infusion.

In replacing the infusion broth, previously recommended by the National Institute of Health, with the fluid thioglycollate medium two distinct advantages were apparent: (1) neutralization of the bacteriostatic action of the mercurial preservatives and (2) provision of both aerobic and anaerobic conditions in one test tube. The advantage of the neutralization of the bacteriostatic effect of the mercurial compounds has been emphasized by Nungester *et al.* (1943). The varying oxidation-reduction conditions in a single culture tube renders the medium valuable for diagnostic use (Foley and Schaub, 1944).

Subsequent use of the thioglycollate medium for the sterility test demonstrated that it was superior to the infusion broth. In parallel tests with the two media, Dr. H. C. Batson (personal communication) detected the presence of many more contaminants with the use of the new medium. A few contaminants, however, developed only in the broth. He found that the latter were inhibited by the methylene blue used as an Eh indicator in the thioglycollate medium. Dr. Geoffrey Edsall (personal communication) reported similar observations; and we confirmed their findings. This inhibitory action could be reduced but not entirely eliminated by enrichment of the medium.

The purpose of the present investigation was to develop a formula for the sterility test medium which would support the growth of the greatest possible variety of bacteria when cultured at the optimum temperature. In accomplishing this it was hoped that it would be possible to use only ingredients lending themselves readily to exact definition and reproducibility, and obtainable without restriction on the open market. Particular emphasis was placed upon the choice of a peptone and an oxidation-reduction indicator, as well as the determination of the optimum amount of yeast extract, sodium chloride, and *L*-cystine, and also the most favorable pH.

During the investigation many people were consulted; from them valuable suggestions and assistance were received. To all are expressed thanks and appre-

ciation. Among those deserving special mention are Dr. John H. Brewer for his constant interest, particularly in reference to the peptone and the chemical tests for the purity of sodium thioglycollate and thioglycollic acid; Dr. J. Howard Brown for tests to determine the digestion products and the growth-promoting action of the pancreatic digest of casein (peptone); Mr. H. G. Dunham, of Difco Laboratories, for preparation of some experimental media and for the suggestion to use resazurin; Mr. Theo. J. Carski, of Baltimore Biological Laboratory, for preparation of certain experimental media; Dr. Harriette D. Vera for the suggestion to use *l*-cystine; Dr. J. G. Brereton, of Sheffield Farms Company, for interest in the production of pancreatic digest of casein; Mr. B. A. Linden for suggestions and co-operation in some duplicate tests; and Dr. Milton V. Veldee for assistance in planning the work.

METHODS

Media. The formulae of the experimental media or the variations from the final revised formula are given with the protocols of the experiments. The revised formula as finally adopted is as follows: *l*-cystine, 0.75 g; sodium chloride, 2.5 g; glucose, 5.0 g; agar, 0.75 g; water-soluble extract of yeast, 5.0 g; pancreatic digest of casein (peptone), 15.0 g; sodium thioglycollate, 0.5 g, or thioglycollic acid, 0.3 ml; resazurin, 1.0 ml of a 0.1 per cent solution freshly prepared; and distilled water, 1 L.

When *l*-cystine was first included in the experimental media, some difficulty was experienced in dissolving it. This was overcome by mixing the dry ingredients, except the sodium thioglycollate, in a mortar in the order given above. Each was thoroughly mixed as it was added. Then a portion of the water, previously heated, was added with stirring, and the resulting paste transferred to a suitable flask. The remainder of the water was added. The solution was completed in a steam bath. Then the sodium thioglycollate or thioglycollic acid was added, the pH was adjusted with sodium hydroxide, and finally the resazurin was added. The medium was dispensed in 15-ml amounts in 20 × 150-mm test tubes and sterilized in the autoclave for 18 minutes at 120 to 123 C.

The pH of the finished media used in the greater part of the study was 7.2 ± 0.1 . An analysis of many experiments revealed that better growth had been obtained in those media having a pH of 7.1 than in those having a pH of 7.2 or 7.3. This was confirmed by further experimentation.

Cultures. The cultures used in the study either had been isolated from contaminated biological products or were considered as possible contaminants, and were very largely selected for their exacting growth requirements. It is imperative that an all-purpose sterility test medium should be capable of initiating the development of *Clostridium tetani* and other pathogenic anaerobes. Cultures of certain other species of *Clostridium* were also used because of their peculiar growth requirements.

For a particular experiment, the cultures were selected which were the most exacting toward the ingredient under study. If any changes were indicated to

meet the growth requirements of a fastidious organism, it was always ascertained that the most commonly occurring contaminants, gram-positive cocci, diphtheroids, sporeforming aerobes, and bacteria of the coli-aerogenes group and of *Pseudomonas*, would in no wise be hindered in their development by these changes.

A list of the cultures is given below. Some of the cultures have not been fully identified, but they have been placed with that genus or group to which they appear to be most closely related.

<i>Aerobacter</i> <i>cloacae</i>	PC3B	red pigment curved forms	Mich. 14 PC66
<i>Bacillus</i> <i>cereus</i> <i>subtilis</i> *	PC3A Peoria	<i>Micrococcus</i> <i>epidermidis</i> <i>roseus</i> (?) <i>rosaceus</i> (?)	PC8 Mich. 57 Mich. 58
<i>Clostridium</i> * <i>acetobutylicum</i>	824 6085	species (?) no pigment pale yellow pigment	PC99 Mich. 14
<i>butylicum</i>	37 Yale	pink pigment orange-pink pigment lemon-yellow pigment	Mich. 38 Mich. 54 Mich. 60
<i>chauvoei</i>	Mont. 2585	<i>Micromonospora</i>	
<i>novyi</i>	140 N.I.H.		Mich. 48 Mich. 53
<i>perfringens</i>	SR12	<i>Diplococcus pneumoniae</i> * type 3 type 17 type 37	
<i>sporogenes</i>	N I.H.		
<i>tetani</i>	Tullock	<i>Pseudomonas</i> <i>ovalis</i>	PC25
<i>Corynebacterium</i> <i>pseudodiphtheriticum</i> *	(Mass.)	species (?) species (?)	PC84 I60 (Mass.)
species (?)	PC20 PC32 PC33B PC96 Mich. 61	<i>Staphylococcus</i> <i>aerogenes</i> <i>aureus</i> * <i>epidermidis</i>	PC1 Cumming PC63
Gram-positive rods, genus (?)		<i>Streptococcus</i> <i>liquefaciens</i> <i>pyogenes</i> * species (?) species (?)	PC16 NY5 PC22 PC33A
orange-pink pigment	Mich. 6		

* Not isolated from contaminated biological products.

Preparatory to inoculating the media the aerobic cultures usually were grown on agar slants; a few were grown in broth. The anaerobic cultures either were grown in a cooked meat medium or a fluid thioglycollate medium. Overnight incubated cultures were used when possible; some required longer incubation. Suspensions of the agar slant cultures as well as the other cultures were diluted tenfold serially in 0.4 or 0.85 per cent sodium chloride solution until just less than one bacterium was present per ml. The highest 5 or 6 dilutions were used for inoculating each medium under test. The inoculum was 1.0 ml per tube. After inoculation the tubes were twirled for thorough mixing and then incubated at 34 to 36 C or at room temperature for 7 days. Two *Pseudomonas* cultures grew very poorly at 34 C and not at all at 37 C. Examination of the tubes for visible growth was made at the end of 1, 2, 4, and 7 days. The degree of turbidity was recorded as 1+, 2+, 3+, and 4+.

EXPERIMENTAL

Eh Indicators

The bacteriostatic or inhibitory action of methylene blue against 8 cultures is shown in table 1. In columns 2 to 5 are recorded the number of tubes, inoculated with 6 dilutions of the respective bacterial suspensions, which showed growth of the bacteria in the medium without and with indicators. There was growth in a total of 28 tubes of medium containing no indicator (column 2) in contrast to a total of 8 tubes of the same medium to which methylene blue had been added (column 3). The same degree of inhibition was obtained in the presence of the zinc chloride salt of methylene blue (see columns 4 and 5). In contrast, there was no inhibition in the presence of resazurin, as shown in column 6.

The bacteria of a large number of species were cultured in the presence of resazurin. Growth was the same as in the medium containing no resazurin, excepting with a few with which the results were equivocal. The latter were cultured in concentrations of resazurin varying from 1:1,000,000 to 1:100,000. A summary of these results is given in table 2. The aerobic cultures were not inhibited in concentrations of 1:250,000 or less, but in 1:100,000 there was retardation in development. On the other hand, the anaerobic cultures were not retarded in the highest concentration, 1:100,000; in fact, some were definitely stimulated by the greater amounts of resazurin. These results suggest that there would be no contraindication to the use of resazurin in a concentration of 1:250,000 or less. A concentration of 1:1,000,000 seemed to be sufficient for indicating the oxidation-reduction conditions of the medium. This amount was less than one-fourth of that which inhibited the development of the most sensitive bacteria that we tested.

It should be mentioned that resazurin in a neutral solution is blue. In the presence of a reducing agent it is reduced to resorufin, which gives a pink color, and resorufin is reduced to colorless hydroresorufin. The latter reaction is reversible; therefore, in the medium containing thioglycollate the reduced portion is colorless, whereas the oxidized portion is pink. An attempt was made to use resorufin in place of resazurin but we obtained a brownish red color which was

more difficult to detect than the pink obtained from resazurin. There also was doubt about its chemical purity even though it was equally noninhibitory.

TABLE 1
Bacteriostatic action of Eh indicators

CULTURE	NO INDICATOR				METHYLENE BLUE				METHYLENE BLUE ZINC CHLORIDE								RESAZURIN			
									9781				140582							
	Day																			
	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7
Mich. 6 .		3*	4	4														3	5	5
Mich. 14 .	2	4	4	4		2	3	3		1	3	3		1	2	3	2	3	4	4
Mich. 38 .		3	3	3			1	2			1	2			1	1		3	4	4
Mich. 48 .	2	3	5	5				1				1				1	1	1	4	4
Mich. 53		2	3	3														2	3	3
Mich. 54		1	2	2															2	2
Mich. 57.. . . .		2	2	2				1				1				1	2	3	3	3
Mich. 60	2	4	5	5				1							1	2	2	4	4	4
Total numbers of tubes . .	6	22	28	28		2	4	8		1	4	7		1	4	8	7	19	29	29
Total turbidity values†	9	39	85	98		2	7	17		1	6	13		2	7	15	10	35	83	100

The formula of the medium differed from that of the revised in specifying 0.1 per cent of *l*-cystine and 0.5 per cent of sodium chloride. The methylene blue and resazurin were present in a final concentration of 1:500,000; the zinc salts were added to give a concentration of 1:500,000 of methylene blue.

* Numerals indicate the number of tubes inoculated with tenfold dilutions of the cultural suspension, which had visible turbidity.

† The degree of turbidity of growth was expressed as 1, 2, 3, and 4. The value given in the table is the summation of the values of the respective tubes.

TABLE 2
Influence of resazurin on growth of bacteria

NUMBER OF CULTURES	RESAZURIN															
	None				1:1,000,000				1:250,000				1:100,000			
	Day															
	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7
Aerobic, 7	13.5	26.5	30.5	31.5	11.5	25.0	29.5	31.0	11.5	24.5	30.0	32.0	4.5	10.0	21.0	27.0
Anaerobic, 4	18.5	20.5	21.5	21.5	17.5	18.5	18.5	18.5	17.5	19.5	19.5	20.0	19.5	21.5	21.5	21.5
Total, 11	32.0	47.0	52.0	53.0	29.0	43.5	48.0	49.5	29.0	44.0	49.5	52.0	24.0	31.5	42.5	48.5

The formula of the medium differed from that of the revised in specifying 0.5 per cent of sodium chloride. Each dilution was inoculated into duplicate tubes of medium. The numerals represent the sum of the average of the duplicates which showed visible turbidity.

For several years the use of resazurin in a reduction test to determine the sanitary condition of milk has been gradually replacing the use of methylene blue

(Ramsdell, Johnson, and Evans, 1935; Davis, 1942; Johns, 1942). Because of the need of a pure product for this use, resazurin can now be obtained in a relatively pure state.

Peptone

Peptones derived from casein have been used exclusively in this investigation. Preliminary tests showed that the development of even the fastidious bacteria

TABLE 3
Comparison of pancreatic and acidic hydrolyzates of casein

NUMBER OF CULTURES	TYPE OF HYDROLYSIS	DEVELOPMENT OF BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
20 (Medium 1)	Pancreatin Acid	57	81	89	89	149	240	321	337
		35	67	77	81	91	188	268	291
8 (Medium 2)	Pancreatin Acid	17	35	38	38	44	91	135	150
		15	31	39	40	39	81	129	145
5 (Medium 3)	Pancreatin Acid	9	20	27	27	22	46	81	97
		7	16	22	22	18	33	64	72
Totals: 33	Pancreatin Acid	93	136	154	154	215	377	537	584
		57	114	138	143	148	202	461	508

Media formulae:

	NO. 1	NO. 2	NO. 3
	<i>g per L</i>	<i>g per L</i>	<i>g per L</i>
Casein hydrolyzate	10.0	10.0	10.0
Yeast extract	10.0	10.0	10.0
Glucose	5.0	5.0	5.0
Sodium chloride	5.0	2.5	5.0
Sodium thioglycollate	0.5	0.5	0.5
Agar	0.7	0.8	0.8
Dipotassium phosphate		1.0	
Resazurin	0.001	0.001	0.001

was promoted by these peptones. It is believed by some that a peptone prepared by the pancreatic digestion of milk protein is more uniform in composition than a similar product prepared as a by-product of the meat-packing industry. Nonallergenic peptone can be made without difficulty; this is of prime importance when antigens intended for human parenteral use contain culture medium. Furthermore, it lends itself to a detailed description so that the product can be duplicated at will (Leifson, 1943).

Enzyme versus acid hydrolyzate. Pancreatic or tryptic digests of casein have been found to be more desirable than an acid hydrolyzate (bacto-casamino acids).

The former contain a larger number of amino acids. Of particular note is the presence of tryptophane. The results of three experiments, in which the growth-promoting activity of peptones obtained by means of the two types of hydrolysis was compared, are given in table 3. In each experiment the initiation of growth was earlier in the presence of the pancreatic digest. However, by the end of 7 days the difference was reduced. Nevertheless, in the case of sterility testing when it is most desirable to detect contamination as early as possible, the use of the pancreatic digest would be indicated in preference to the acid hydrolyzate.

Comparison of different preparations of pancreatic digest of casein. The growth-promoting activity of pancreatic or tryptic digests prepared by 5 manufacturers has been studied. The results of 2 experiments are summarized in table 4. It is apparent that when the respective peptones were used in combination with the other ingredients of the medium, there was no significant difference in their promotion of the growth of the exacting bacteria which had been selected for

TABLE 4
Pancreatic digests of casein prepared by different manufacturers.

NUMBER OF CULTURES	DESIGNATION OF PEPTONE	DEVELOPMENT OF BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
11*	A	18	32	42	42	45	87	144	161
	B	17	33	38	42	50	99	143	151
	C	18	29	37	40	44	91	131	147
	D	16	30	43	44	43	84	151	165
10†	A	23	39	45	48	44	107	152	167
	E	19	36	43	46	39	97	144	168

* The formula of the medium differed from that of the revised in specifying 0.1 per cent of *l*-cystine and 0.07 per cent of agar.

† Revised formula.

these experiments. However, in other experiments, in which the yeast extract was omitted from the medium, certain preparations were definitely better than others.

Yeast Extract

The addition of a water-soluble extract of yeast definitely favored the development of the bacteria. The results of 2 experiments are given in table 5. In the first the growth of bacteria in a medium containing 2.0 per cent of peptone was compared with that in a medium containing 1.0 per cent of peptone and 1.0 per cent of yeast extract. In the presence of the latter there was growth at the end of 24 hours in 10 more tubes, or 21 per cent, than in the former; and at the end of 48 hours the difference had increased to 20 tubes, or 33 per cent.

In the other experiment 3 concentrations of yeast extract, 0.2, 0.5, and 1.0 per cent, were used in combination with 2.0, 1.5, and 1.0 per cent of the peptone,

respectively. The best development was obtained in the presence of 0.5 per cent yeast extract and 1.5 per cent peptone. These results are not significantly different from those obtained with the medium containing 1.0 per cent of yeast extract and 1.0 per cent of peptone. It is definite, however, that in the presence of 0.2 per cent of the yeast extract and 2.0 per cent of peptone there was a lag in the initiation of growth. We have observed no inhibition in development in the presence of 1.0 per cent of the yeast extract, but Dr. G. B. Slocum (personal communication) has noted that *Lactobacillus* is inhibited in the presence of more than 0.5 per cent. It appeared that 0.5 per cent of yeast extract would be an adequate and satisfactory amount to use in the fluid thioglycollate medium. No doubt the extract supplies certain growth factors which aid in the promotion of the development of bacteria. Leifson (1943) has reported that the addition of

TABLE 5

Influence of varying amount of yeast extract in the presence of different amounts of peptone

NUMBER OF CULTURES	VARIATIONS IN MEDIA		DEVELOPMENT OF BACTERIA—DAY							
	Peptone	Yeast ext.	Number of tubes				Turbidity values			
			1	2	4	7	1	2	4	7
20	%	%								
	2.0	0.0	47	61	73	74	131	176	251	270
	1.0	1.0	57	81	89	89	149	240	321	337
21	2.0	0.2	34	64	77	81	110	193	246	276
	1.5	0.5	44	72	80	83	121	214	267	300
	1.0	1.0	44	67	77	80	121	211	267	295

Formula of basic medium: For amounts of casein peptone and yeast extract see above; glucose, 0.5%; sodium chloride, 0.5%; *l*-cystine, 0.1%; sodium thioglycollate, 0.05%; agar, 0.07%; and resazurin, 0.0001%.

accessory growth factors greatly improve the general nutritive properties of casein peptones.

Buffer

It is shown in table 6 that more rapid development of the bacteria took place in the absence of the buffer (K_2HPO_4) than in its presence. At the end of 24 and 48 hours there was growth in 10 (29 per cent) and 16 (31 per cent) more tubes, respectively. However, at the end of 7 days there was growth in the same number of tubes of each medium. The effect of the buffer seemed to be retardation. In this experiment the buffer was added to adjust the pH, and 0.3 per cent was required. This amount is slightly in excess of the 0.25 per cent that was specified in the first formulae of the fluid thioglycollate media recommended by the National Institute of Health. It may be that this additional amount of the potassium ion acted as an inhibitor. Since the amount of buffer originally used is not sufficient to neutralize the acidity that might be produced from the

0.5 per cent glucose, and its presence is not needed for the development of the bacteria, it seemed better to omit it from the formula.

Sodium Chloride and Hydrogen Ion Concentration

Bacteria of different species vary in their requirements for sodium chloride and hydrogen ion concentration in order to attain optimum development. In table 7 it is shown that *Clostridium acetobutylicum*, 824, developed best in the medium with the lowest pH used, 7.1, to which no sodium chloride had been added. The pH of the medium was more influential than the amount of sodium chloride. This is graphically illustrated in figure 1. Here it may be seen that this culture developed in the presence of 0.5 per cent of sodium chloride at a pH of 7.11, although there was some retardation. On the other hand, in the

TABLE 6
Influence of buffer, dipotassium phosphate

NUMBER OF CULTURES	BUFFER K ₂ HPO ₄ %	DEVELOPMENT OF THE BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
21	0.3	34	51	72	80	100	169	223	276
	None	44	67	77	80	121	211	267	295

Formula of basic medium: Casein peptone, 1.0%; yeast extract, 1.0%; glucose, 0.5%; sodium chloride, 0.5%; L-cystine, 0.1%; sodium thioglycollate 0.05%; agar 0.07%; and resazurin 0.0001%.

medium with a pH of 7.35, to which no sodium chloride had been added, there was very little development. The Yale strain of *C. acetobutylicum* was not so sensitive to variations in pH.

In contrast it is shown, also in table 7, that the Montana strain of *Clostridium chauvoei* developed better in the presence of sodium chloride than in its absence, although there was a tendency for it to favor the low pH. This preference may be observed best by comparing the turbidity of growth at the end of 1 and 2 days in the presence of the different salt concentrations at the different pH values. The other strain of *C. chauvoei* studied behaved similarly.

Differing from the requirements of both *C. acetobutylicum* and *C. chauvoei* were those of the type 3 pneumococcus (table 7). It developed best in the presence of the highest salt concentration and highest pH that were employed, 0.5 per cent and 7.32, respectively. Other strains of the pneumococcus were not so sensitive to variations. However, with the fluid thioglycollate medium the optimum pH for the pneumococcus seemed to be between 7.2 and 7.3 and the presence of sodium chloride was indicated as desirable.

In another experiment it was observed that the *Micrococcus*, Mich. 38, and the *Micromonospora*, Mich. 53, developed better at pH 7.4 than at 7.2 or 7.0. The presence or absence of sodium chloride did not appear to be significant.

In order to promote the development of bacteria which vary in their requirements for sodium chloride and hydrogen ions, a mid-course was followed and a concentration of 0.25 per cent sodium chloride and a pH of 7.1 ± 0.1 were

TABLE 7

The influence of sodium chloride in the presence of varying hydrogen ion concentrations

CULTURE	SODIUM CHLORIDE	pH	DEVELOPMENT OF THE BACTERIA—DAY							
			Number of tubes				Turbidity values			
			1	2	4	7	1	2	4	7
<i>C. acetobutylicum</i> , 824	0.5	7.32								
		7.11		3	4	4		11	16	16
		7.0	3	4	4	4	7	16	16	16
	0.25	7.32								
		7.11	3	4	4	4	6	16	16	16
		7.0	4	4	4	4	13	16	16	16
		7.35		1	1	1		4	4	4
		7.18	4	4	4	4	11	16	16	16
		7.1	5	5	5	5	15	20	20	20
<i>C. chauvoei</i> , Mont.	0.5	7.32	3	4	4	4	3	11	16	16
		7.11	3	3	3	3	5	11	12	12
		7.0	3	3	3	3	9	11	12	12
	0.25	7.32	2	3	3	3	2	4	11	12
		7.11	2	3	4	4	2	8	15	16
		7.0	4	4	4	4	7	16	16	16
		7.35		1	3	3		1	9	12
		7.18	1	2	3	3	1	3	8	12
		7.1	1	1	5	5	1	3	10	20
<i>Diplococcus pneumoniae</i> , type 3	0.5	7.32	4	4	5	5	7	12	17	20
		7.11	3	5	5	5	5	13	20	20
		7.0	3	5	5	5	4	5	17	20
	0.25	7.32	3	3	4	4	3	3	16	16
		7.11	3	3	4	4	4	8	13	16
		7.0	2	4	4	5	2	8	16	16
		7.35		1	3	3		2	7	12
		7.18	1	1	3	3	1	2	10	12
		7.1	1	1	3	3	1	3	9	12

Revised formula.

selected for the medium. It should be mentioned that the ingredients used in the preparation of the medium must have a uniformly low sodium chloride content.

Besides the bacterial need for sodium chloride, the presence of the salt in the sterility test medium may be necessary for the development of contaminants in some biological products containing mercurial preservatives. In one of the early experiments when many variations were being studied, 14 different media were

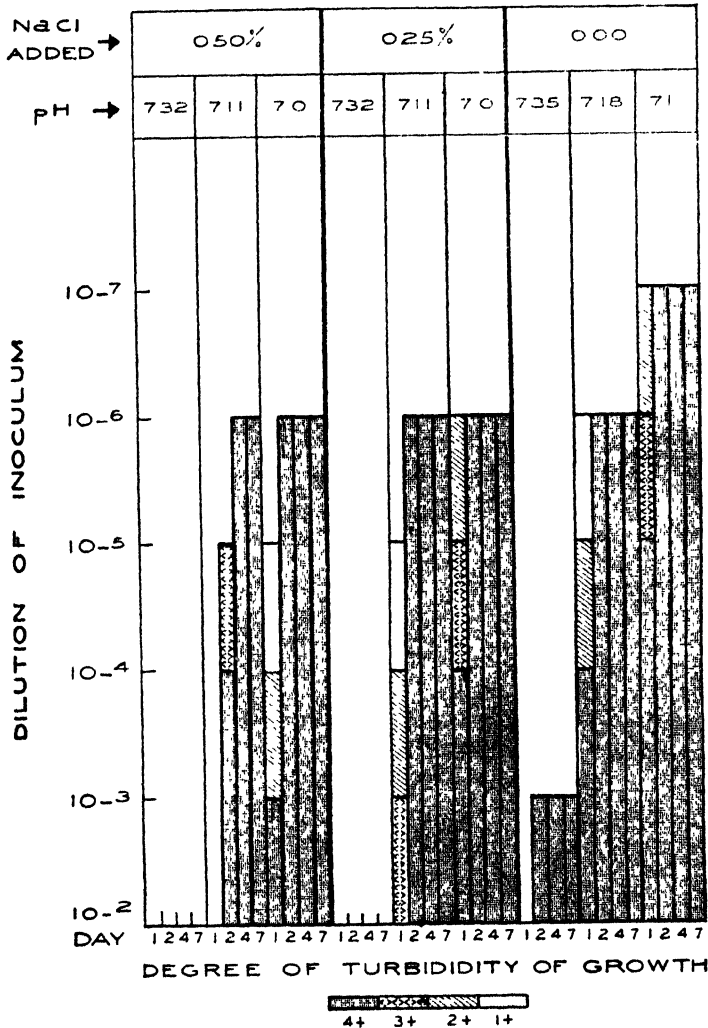


FIG. 1. THE GROWTH OF *C. ACETOBUTYLICUM*, STRAIN 824, IN THE PRESENCE OF VARYING AMOUNTS OF SODIUM CHLORIDE AND DIFFERENT pH CONCENTRATIONS

under test. Several contaminated products were seeded into the media. With a normal serum albumin solution containing 1:10,000 merthiolate, it was observed that in 7 of the media no growth developed from the inocula of the undiluted and the 10⁻¹ dilution, but excellent growth developed from the 10⁻², 10⁻³, and 10⁻⁴ dilutions. In the other 7 media there was development from all 5 inocula.

The media differed considerably in composition. The only common factor in the first seven was that no sodium chloride had been added. Salt had been added to the other media although only 0.15 per cent had been added to two. It is thought that perhaps the salt combined with the mercurial compound to form a less soluble product, thereby permitting the bacteria to grow. The contaminant was a diphtheroid. Dilutions of a suspension of this culture from agar initiated growth in all 14 media.

The presence of sodium chloride was not the controlling factor in the development of the bacteria in the other biological products tested at the same time.

l-Cystine

C. chauvoei does not develop in fluid thioglycollate medium without the addition of *l*-cystine. The chances for this organism to contaminate a biological

TABLE 8

Influence of varying amounts of l-cystine on the development of C. chauvoei Mont.

DILUTION OF INOCULUM	AMOUNT OF L-CYSTINE—PER CENT														
	0.1			0.075			0.05			0.025			None		
	Day														
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
10 ⁻²	4	4	4	4	4	4	3	4	4	3	4	4			3
10 ⁻³	4	4	4	4	4	4	4	4	4	1	2	4			
10 ⁻⁴	4	4	4	4	4	4	2	4	4	2	3	4			
10 ⁻⁵	4	4	4	4	4	4	3	4	4		3	4			
10 ⁻⁶	4	4	4	4	4	4		4	4		3	4			
10 ⁻⁷															

The medium was prepared according to the revised formula with variations in the amount of cystine. Numerals indicate degree of turbidity.

product prepared for human use are remote. On the other hand, if the medium is to be used in connection with products for veterinary use, it would be necessary for it to be adequate for the development of this organism. So far as we have been able to determine, there is no contraindication to its inclusion and it is probable that its presence may stimulate the development of other bacteria.

From the results of the experiment reported in table 8 it appears that 0.075 per cent of *l*-cystine is sufficient to obtain the most rapid growth of *C. chauvoei*; 0.05 per cent would probably be sufficient, but with less than 0.05 per cent there is a lag. Note how this organism failed to grow in the medium containing no *l*-cystine.

Shortly after the official date of adoption of the revised formula (January 15, 1945) Hickey suggested the inclusion of cysteine hydrochloride in fluid thioglycollate medium for the sterility testing of penicillin. Since *l*-cystine had been included in the medium, it was thought that perhaps part of the *l*-cystine would be reduced to cysteine in the presence of the thioglycollate and that the new

medium might be suitable for the sterility testing of penicillin without the addition of an inactivating agent. Hewitt and Pittman (1945) found, however, that there was no inactivation of penicillin in the medium in a ratio of 10 units to 0.75 mg of *l*-cystine within 3 hours.

Temperature for Incubation

Sufficient experimentation has not been done to determine the optimum temperature for incubation of the sterility test. However, so far as the work has gone, it is indicated that 32 C might be favorable to all contaminants. One experiment was conducted in duplicate with incubation at 36 C and at 22 to 25 C. All of the cultures grew at the lower temperature, whereas one did not grow, and others grew more poorly, at the higher temperature. Miss Frances Clapp, of Lederle Laboratories, has reported to us the isolation of contaminants with incubation at 31 C when no growth was obtained with incubation at 37 C. On the other hand, she has obtained no growth at 37 C that she has not likewise obtained at 31 C.

So far as we know there are no bacteria that might occur as contaminants that develop at 37 C and will not likewise develop at 31 or 32 C, whereas there are a number which will not grow at 37 C. In the latter group there are some of the *Pseudomonas* cultures, and these are notorious for their ability to be pyrogenic (Probey and Pittman, 1945).

SUMMARY

A study has been presented which formed the basis for the adoption of a revised formula for the "Fluid thioglycollate medium"; this formula replaces the two previously recommended for use in the testing of biological products for sterility. It was desirable that all ingredients included in the formula should lend themselves to full description. Such descriptions in addition to full directions for preparing the medium and its use are given in the National Institute of Health circular, "Culture Media for the Sterility Test," dated January 15, 1945, and it is anticipated that they will be included in the thirteenth edition of the *U. S. Pharmacopoeia*.

Resazurin was found to be a suitable Eh indicator. In concentrations of 1:250,000 or less, resazurin did not retard the development of the bacteria tested, whereas methylene blue in a concentration of 1:500,000 was definitely bacteriostatic for certain bacteria. A 1:1,000,000 dilution of the resazurin is sufficient to indicate oxidation-reduction conditions.

Pancreatic digest of casein is apparently an adequate peptone for the sterility test medium. It has excellent growth-promoting properties which should make it useful in many kinds of media. In its presence, initiation of growth was more rapid than in the presence of an acid hydrolyzate of casein.

A water-soluble extract of yeast was influential in promoting growth of the bacteria. The optimum amounts of the extract and the casein peptone appear to be 0.5 and 1.5 per cent, respectively.

Dipotassium phosphate tended to retard initiation of growth.

A pH of 7.1 and the addition of 0.25 per cent of sodium chloride seem to provide suitable conditions for the development of those bacteria that prefer a low pH and no sodium chloride and those that prefer a higher pH and 0.5 per cent of sodium chloride.

The addition of *l*-cystine enables *Clostridium chauvoei* to develop in the medium.

The optimum temperature for incubation of the sterility test is discussed.

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LIPID PRODUCTION BY A SOIL YEAST

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It has been well established that fatty substances are produced by various microorganisms, notably by certain yeasts and filamentous fungi as well as by the tubercle bacilli and a species of *Azotobacter*. The tubercle bacilli and other acid-fast bacteria contain from 20 to 40 per cent lipid on the dry-weight basis (Anderson, 1939). Even some of the common non-acid-fast bacteria may contain considerable amounts of lipid. *Escherichia coli*, *Staphylococcus albus*, and *Bacillus megatherium* were found to contain from 8 to 40 per cent lipid when grown on certain media (Larson and Larson, 1922). More commonly, however, the lipid content of bacteria has been reported to be below 10 per cent. The results of Gorbach and Sablatnög (1934a, 1934b) may be cited in this connection. They found that whereas the lipid content of *Pseudomonas aeruginosa* was 0.6 per cent when grown on meat extract agar at pH 7.0, there was as much as 3.9 per cent lipid when the organism was cultivated on mannitol. Anderson (1939) reported 2 to 6 per cent lipid in cells of *Phytomonas tumefaciens* and 7 per cent in *Lactobacillus acidophilus*.

Among the bacteria, *Azotobacter indicum* is unique in lipid production (Starkey and De, 1939). The cells of this bacterium commonly contain two large fat globules, one at each end of the rod-shaped cells, and as much as 50 per cent of the cell volume is occupied by the fat globules.

The lipid content of filamentous fungi ranges between 1 and 40 per cent, and differs with the various cultures and the conditions under which they are grown. (Pruess, Eichinger, and Peterson, 1934; Prescott and Dunn, 1940; Bloor, 1943). The lipid content of 24 filamentous fungi studied by Pruess and Strong (1933) was from 1 to 25 per cent with an average between 6 and 9 per cent. Ward, Lockwood, May, and Herrick (1935) determined the lipid content of 61 fungi and reported that six of the fungi contained over 20 per cent lipid. Under favorable conditions of cultivation, *Penicillium javanicum* was found to contain as much as 41.5 per cent. Large amounts of lipid were produced by species of *Oospora* Wallroth when grown on milk, and the results of Geffers (1937) indicate that this organism can be used to synthesize fat from milk wastes. Up to 50 per cent of the mycelium could be extracted with fat solvents.

Attention was focused on lipid production by yeasts and related organisms through the results of Lindner and associates, who developed a process for the production of fat from carbohydrates by *Endomyces vernalis* in Germany during World War I (Fink, Hahn, and Hoerbuerger, 1937; Prescott and Dunn, 1940). When cultivated under conditions favorable for lipid production, this yeast con-

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tained as much as 44.7 per cent lipid; but when cultivated on a production scale, there was about 20 per cent lipid. Heide (1939), who recently studied the influence of medium composition on lipid production by *Endomyces vernalis*, found as much as 42 per cent lipid in the cells under conditions favorable for lipid accumulation.

Several yeasts were studied by Rippel (1943) for lipid production, among which was *Nectaromyces reukaufii*, which produced 10 to 15 g of lipid for each 100 g of carbohydrate decomposed.

The results of Heide (1939) and others (Bichkovskaya, 1939; Prescott and Dunn, 1940; Raaf, 1941-1942; Rippel, 1943) indicate that conditions favorable for maximum lipid content of yeast are not those giving the maximum conversion of the carbon source of the substrate to lipid. Cells grown in media rich in nitrogen give high yields, but their lipid content is low; media deficient in nitrogen give low yields of cell material, but the cells contain an abundance of lipid. As stated by Smedley-MacLean (1922), there is generally an inverse relationship between the quantity of yeast cells produced on various media and the percentage of lipid in the cells.

The relative concentrations of carbohydrate and nitrogenous materials in the media must therefore be carefully balanced to obtain the maximum yield of lipid. Among the other factors affecting lipid production are the reaction of the medium, phosphate concentration, degree of aeration, nature of the carbon source, and the presence of certain metallic ions. Not only are there great differences in the potentialities of different microorganisms for lipid production but the composition of the medium and the environmental conditions likewise have a profound effect on lipid production by any one culture. The substrates which have been proposed for lipid production by yeasts and filamentous fungi include milk waste, molasses, waste sulfite liquor, cellulose waste, and hydrolyzed wood.

Methods have also been developed for the production of yeast as a source of protein. The yeast is cultivated in media containing carbohydrate and supplied with nitrogen from ammonium salts. Thaysen (1943) and Thaysen and Morris (1943) recently reported results of studies with *Torulopsis utilis* from which it was concluded that this "food yeast" which was rich in protein and vitamins could be produced very economically (Gortner and Gunderson, 1944). New strains were developed which grew well at relatively high temperatures and produced cells larger than the original strains, thus facilitating production and processing.

EXPERIMENTAL

The yeast with which this report is concerned was encountered during studies of the nitrogen-fixing population of soils and has been recovered from soils periodically since 1935.¹ The yeast cultures were obtained from colonies which developed on a "nitrogen-free" agar medium having the following composition: glucose, 15 g; K_2HPO_4 , 0.8 g; KH_2PO_4 , 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g;

¹ A preliminary report was presented before the Theobald Smith Society (Starkey, 1944).

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0005 g; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.0005 g; $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 0.0005 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g; agar, 15.0 g; and distilled water, 1,000 ml. A small amount of the soil being tested was scattered over the surface of plates of the solidified agar medium and incubated at 28 C. The common types of *Azotobacter*, if present, usually developed within 2 to 3 days and were characterized by their glistening, soft, smooth, raised colonies. When the incubation period was prolonged to from 7 to 10 days, other colonies appeared which resembled those of *Azotobacter*. They were raised and glistening and had the appearance of very thin starch paste. They were watery, tended to spread, and sometimes coalesced, forming a thin watery film over a considerable portion of the plate. When examined under the microscope, this material was found to contain large, nearly spherical cells approximately 8 μ in diameter suspended in thin slime. Some of the cells had a single smaller spherical bud, but there was no tendency toward the formation of large aggregates. Each of the cells was found to contain a large, highly refractive globule which nearly filled the cell (figures 1, 2, and 3).

The watery, spreading colonies were consistently obtained from various soils including those from cultivated and uncultivated fields, from pastures, waste land, and woodland. The soils varied in reaction from pH 4.0 to 7.5. The cells which composed the colonies all had the same appearance and were characterized by the presence of large refractive globules, one in each cell. The regularity with which the yeast was encountered suggests that the organism is a common soil inhabitant.

When isolated in pure culture and cultivated on slants of the glucose, nitrogen-free, agar medium, the yeast cultures continued to produce large spherical cells nearly filled with the large refractive globules. The cell material had a watery consistency and tended to settle to the base of the slant.

The appearance of the globule suggested that it was lipid, and tests indicated that this was the case. When the cell material was treated with Sudan III, the globules stained red, whereas the remainder of the cell was colorless. The lipid globules of cells which were treated with warm dilute acid to hydrolyze the slime stained more readily. The fact that large amounts of fatty material were obtained by extracting the cells with fat solvents, as well as the fact that the cells no longer became colored with Sudan III after the extraction, proved that the globules were lipid.

Sporulation. The yeast is of interest not only by reason of its high lipid content but because of its unusual spore formation. The following sequence of changes was observed with cultures developing in association with other microorganisms on the glucose, nitrogen-free, agar medium which had been inoculated with soil. During the first two weeks the cells consisted entirely of the spherical cells with spherical buds, nearly filled with globules of lipid. During the next few days some of the yeast cells produced buds of a different type. They were shaped like a sac and contained material which had a granular appearance in contrast to the homogeneous, refractive lipid globules in the spherical buds (figures 4 and 5). In a few more days the granular substance became trans-

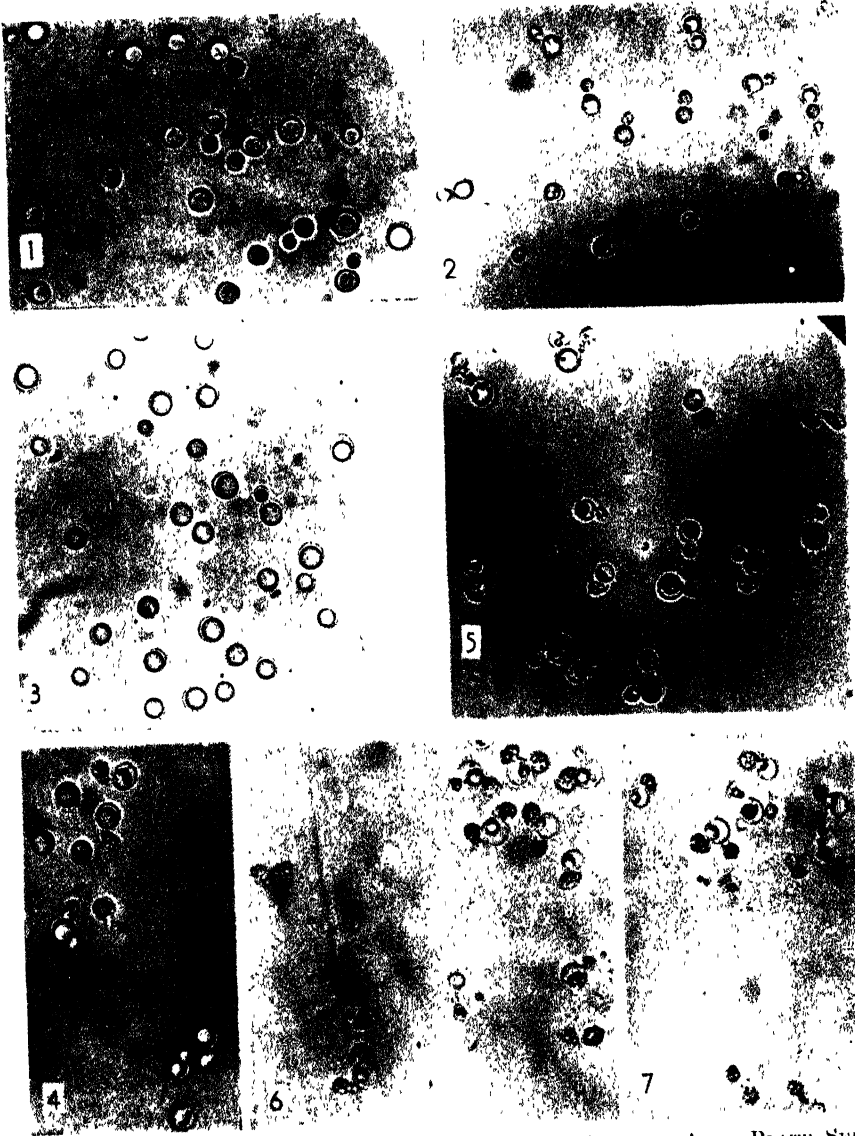


FIG. 1. CELLS FROM A COLONY ON A NITROGEN-FREE, GLUCOSE, AGAR PLATE SEEDED WITH SOIL.

Each spherical cell is nearly filled with a single large fat globule. One cell at lower right has a hazy outline caused by the breaking away of an outer cell membrane. Culture age 13 days. Magnification $\times 330$.

FIG. 2. CELLS OF A PURE CULTURE OF STRAIN 72 GROWN ON GLUCOSE, NITROGEN-FREE AGAR

There is a fair-sized single fat globule in each cell. Culture age 6 days. Magnification $\times 330$.

FIG. 3. CELLS OF A PURE CULTURE OF STRAIN 72

Typical spherical cells each nearly filled with a single fat globule. Culture grown on nitrogen-deficient aerated solution medium. The cells were taken from one of the aerated solutions used for tests of fat production by the yeast. Culture age 20 days. Magnification $\times 330$.

formed into ascospores (figures 6, 7, 8, and 9). The ascus remained attached to the parent cell even after spore formation was completed, and additional vegetative buds were frequently seen attached to the parent cell. In some cases the parent cell bore more than one ascus. In no case was there evidence of spores within the parent cell. The globule of lipid persisted in the parent cell throughout the period of spore formation, but it became reduced in size as the incubation period was prolonged.

Many yeasts, two of which were characterized by the presence of large fat globules, were isolated from soil by Starkey and Henrici (1927). These were described as *Nadsonia fulvescens* and *Torulasporea* sp. The descriptions of these do not characterize the yeast under consideration.

Spore formation has not been observed with pure cultures grown on the glucose, nitrogen-free, agar or similar media containing 0.01 per cent yeast extract. The medium containing 0.01 per cent yeast extract was similar to the medium from which the yeast cultures were isolated, but differed in that it contained no sodium chloride, yeast extract, or salts of manganese, molybdenum, and tungsten. The modified medium with 0.01 per cent yeast extract is the one subsequently designated as the nitrogen-deficient medium. Spores were produced, however, on several media. On the vegetable infusion agar of Mrak, Phaff, and Douglas (1942) spores were first noted with cultures 14 days old, and the asci became more numerous during the incubation period of 2 months. No spores were produced by cultures grown in a solution medium of similar composition. Spores were produced by cultures which were heavily inoculated on media unfavorable for growth, such as nitrogen-deficient agar media in which rhamnose, lactose, ethanol, or glycerol was substituted for glucose. On these media there was no apparent growth in some cases and poor growth in others, but spores were produced. Spores were observed with some cultures after 7 days' incubation on media containing rhamnose, lactose, or glycerol, and there was more sporulation after 27 days.

Nickerson and Thimann (1941) found that material produced by *Aspergillus niger* on malt extract medium enhanced conjugation and spore formation by

FIG. 4. CELLS FROM A COLONY ON A NITROGEN-FREE, GLUCOSE, AGAR PLATE SEEDED WITH SOIL

Several of the cells show saclike buds filled with granular material and attached to the parent cells, each of which is nearly filled with a single large fat globule. Some fat-filled cells free from buds. Culture age 17 days. Magnification $\times 330$.

FIG. 5. CELLS FROM THE SAME CULTURE AS FIGURE 4

Many cells show saclike buds filled with granular material and attached to the parent cells, which have large fat globules. Some cells have 2 buds each. Culture age 17 days. Magnification $\times 330$.

FIG. 6. CELLS FROM THE SAME COLONY ON NITROGEN-FREE, GLUCOSE, AGAR PLATE SEEDED WITH SOIL AS THAT FROM WHICH CELLS OF FIGURES 4 AND 5 WERE OBTAINED

The cells show a transition stage in spore formation. Some of the saclike buds are filled with granular material, whereas other buds contain spores, up to 8 in number. Culture age 20 days. Magnification $\times 330$.

FIG. 7. SAME AS FIGURE 6

One cell at upper right shows an elongated ascus with eight easily distinguished spores. Asci attached to other cells show various stages in spore formation. Culture age 20 days. Magnification $\times 330$.

certain species of *Zygosaccharomyces*. Similar material was added to glucose, nitrogen-deficient, solution media which were inoculated with the lipid-producing yeast to test for spore formation. Other solution media received additions of malt extract, yeast extract, and grass extract. In none of these solutions was there evidence of spore formation even after incubation for several months. In some cases solution media supporting growth of the yeast were inoculated with cultures of *Aspergillus* and *Penicillium*, but the associative development of the fungi failed to result in spore formation by the yeast.

Some strains produced 4 spores in each ascus, whereas in other strains there were as many as 8 spores (figures 6 and 7). One strain produced still larger numbers of spores (strain 74). The vegetative cells of this strain were larger than those of the other strains; 16 or more spores have been seen in the asci, and it is probable that the number was greater than this, since it was difficult to determine the exact number of spores which were enclosed in dense aggregates in the asci (figures 10, 11, 12, and 13). The number of spores in each ascus varied as the composition of the medium was changed, but strain 74 generally had larger numbers of spores than the other strains which were studied.

The spores had a light amber color, and the asci showed some contrast in color with the vegetative cells. The spores were oval in shape and smooth on the surface, and had a size of 2.5 to 3.3×3.6 to 4.6μ , with an average of $3.0 \times 4.3 \mu$.

Size and shape of vegetative cells. The vegetative cells varied greatly in size, but when the cultures were grown on a nitrogen-deficient, carbohydrate medium the fully developed cells of most strains had diameters of 8.0 to 9.5μ . One strain (no. 74) consistently produced larger cells, which had diameters of 9.8 to 10.5μ .

Under certain cultural conditions the cultures produced cells which were variable in shape. When cultivated on malt extract agar or on 2 per cent glucose agar containing 0.1 per cent ammonium sulfate or asparagine, the cells were low in fat content. During the first few days of incubation the cells were mostly spherical, but later they showed variation. They were elongated and swollen and varied greatly in size. On all media the cells multiplied by multipolar budding. Cells of cultures grown on nitrogen-deficient, carbohydrate media generally had no more than one bud. The buds freely separated from the cells grown in these media, but on malt extract agar and on agar media containing larger amounts of nitrogen the cells frequently occurred in clusters.

The cells frequently shed their outside membranes. This coating split and was shed in one piece like the coating on a bean seed, with no apparent deterioration of the cell (figure 14).

Mycelium production. The cultures produced no typical mycelium and showed no tendency toward mycelium production on most carbohydrate media. Some rudimentary pseudomycelium was formed, however, in old cultures on agar media containing rhamnose, glycerol, or mannitol. There was good growth on mannitol but very little development on the other two media. The mycelium consisted of short, distorted, hyphal projections from the round cells, and some of these short hyphae were branched.

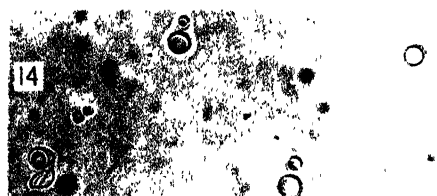
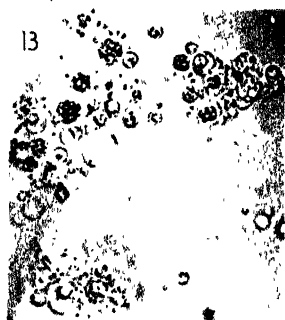
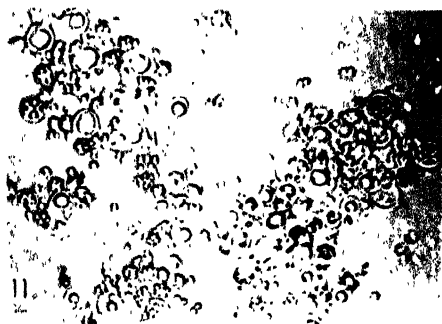
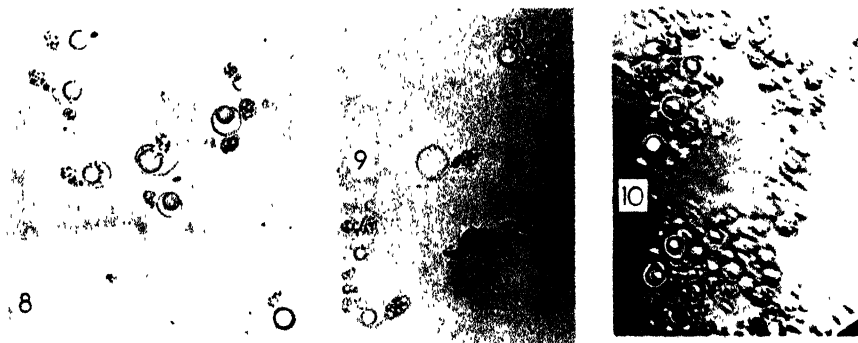


FIG. 8. SAME AS FIGURE 6

One cell in the center shows the initial stage of loss of the outer cell membrane

FIG. 9. SAME AS FIGURE 6

FIG. 10. OLD CELLS OF STRAIN 74 GROWN ON A NITROGEN-DEFICIENT, AGAR MEDIUM CONTAINING 0.5 PER CENT ETHANOL

Most of the vegetative cells had degenerated. Many cells show spores. One cell at upper left shows many spores, 14 of which can be counted. Some cells still show presence of fat. Culture age 28 days. Magnification 330.

FIG. 11. SAME AS FIGURE 10

Some of the cells contain many spores

FIG. 12. SAME AS FIGURE 10

FIG. 13. SAME AS FIGURE 10

FIG. 14. CELLS FROM A NITROGEN-FREE, GLUCOSE, AGAR PLATE

One cell at lower left shows a cap-shaped outer membrane breaking away from the cell. Culture age 13 days. Magnification $\times 330$.

Type of growth on agar and solution media. On glucose, nitrogen-free agar the organism grew slowly and produced glistening, white, watery slime resembling thin starch paste. On a similar medium containing 0.01 per cent yeast extract the cultures grew more rapidly and produced more cell material. On 2.5 per cent malt extract agar there was abundant brownish-gray, raised, spreading, slimy growth. On the plant infusion agar of Mrak *et al.* (1942) the cultures grew very rapidly and produced dark brown, moist, raised, spreading growth. In all cases the cells were imbedded in slime which caused the cell material to settle to the base of the slants. On agar slants in which various carbohydrates were substituted for glucose, the growth characteristics were the same; the cultures produced white, glistening, watery growth having the appearance of starch paste. On glycerol agar the growth was not mucoid but relatively free from slime; the cell material was first white but later turned slightly brown in color. On 2 per cent glucose agar containing 0.1 per cent asparagine there was abundant white, mucoid growth. There was even more growth when 0.1 per cent $(\text{NH}_4)_2\text{SO}_4$ was used as the source of nitrogen, but the cultures produced very little slime on this medium.

The yeast cultures made very poor growth on static solution media; no pellicle was formed and the solutions showed only slight turbidity. Most of the cell material accumulated as sediment. Since development of the organism is characterized by oxidative and not fermentative changes, the submerged cells grew slowly. Growth was very much increased by aeration, and in aerated culture solutions the yeast cells were uniformly dispersed and produced a white to cream-colored turbidity. These results are in agreement with those of Smedley-MacLean and Hoffert (1923, 1926), who reported that lipid storage is favored by vigorous aeration. Their experiments are not strictly comparable, however, since they studied the production of lipid by yeast cells suspended in solutions containing various carbon sources but no nitrogen, and under these conditions growth was suppressed almost completely.

Utilization of carbohydrates and some related compounds. The ability of the yeast to use various substances as energy sources was tested on both agar and solution media having a composition similar to that of the nitrogen-deficient medium but with various carbon compounds substituted for the glucose. The cultures were examined at various periods during the 26 days of incubation. Growth was meagre on all the solution media, but it was more abundant on the agar slants. The same substances supported growth on both agar and solution media. The yeasts grew well on agar slants containing glucose, fructose, galactose, sucrose, mannose, maltose, dextrin, starch, inulin, mannitol, and ethanol. The yeast grew poorly on glycerol. Two of the four strains tested made a small amount of growth on rhamnose; the other two failed to grow. None of the strains grew on lactose.

Glucose was not fermented. Presumably, therefore, none of the carbohydrates is fermented (Henrici, 1941).

Utilization of nitrogen compounds. Although the cultures were isolated from media to which no nitrogen had been added (glucose, nitrogen-free agar), the yeast was unable to fix atmospheric nitrogen. It was cultivated on nitrogen-free

solution media containing 1.5 per cent glucose or mannitol as sources of carbon and on similar media containing 0.01 per cent yeast extract. Some of the cultures were aerated and others were static. In none of the media was there any nitrogen fixation by the yeast, although there was fixation of 9 to 20 mg of nitrogen by cultures of *Azotobacter* grown in 100-ml portions of similar media.

The yeast utilized ammonium salts, asparagine, and yeast extract as sources of nitrogen. Nitrate was not utilized.

Gelatin was not liquefied in gelatin stabs containing 2 per cent glucose, and there was no decomposition of gelatin in gelatin-agar plates containing 2 per cent glucose (Frazier, 1926) during 18 days of incubation.

Influence of medium composition on lipid production. The lipid content of the cells was greatest when the cultures were grown on nitrogen-deficient carbohydrate media. The lipid globules were small in cells grown on malt extract agar, the vegetable infusion agar of Mrak *et al.* (1942), and agar media containing 2 per cent glucose and 0.1 per cent asparagine or $(\text{NH}_4)_2\text{SO}_4$. As has been repeatedly noted by others with cultures of yeast and filamentous fungi, the nitrogen content of the medium had a pronounced effect on lipid production. In media with relatively large amounts of available nitrogen per unit of carbohydrate, lipid accumulation was suppressed. Nitrate had no effect on lipid accumulation. This apparent anomaly is explained by the fact that the yeast did not utilize nitrate. It has been observed that the cells frequently contained smaller fat globules during the first few days of incubation than at later stages of growth.

Cells in static solution media contained relatively small amounts of lipid, whereas they were nearly filled with lipid when cultivated in aerated media of the same composition. The cultures grew well and produced large amounts of lipid in aerated solution media containing 3 per cent glucose, 0.05 per cent $(\text{NH}_4)_2\text{SO}_4$, and 0.01 per cent yeast extract.

All of the yeast strains which have been isolated appeared to be the same except for minor differences. The yeast is characterized by slow growth in solution media, lack of fermentation, distinctive means of sporulation, and high lipid production under suitable cultural conditions.

Classification. The morphological and physiological characteristics of the yeast are such that the organism is not readily classified. Wickerham concluded that, except for spore formation, it is in many respects similar to some members of the asporogenous yeasts of the genus *Torulopsis* (Wickerham, 1944).

A culture isolated by den Dooren de Jong (1926) and named *Torula lipofera* resembles the yeast morphologically and physiologically, except for spore formation. The name of the organism was subsequently changed to *Torulopsis lipofera* by Lodder (1934). The yeast was isolated from soil, produced cells with large fat globules when grown on suitable media, did not ferment carbohydrates, produced no pellicle, and grew on similar carbohydrates, alcohols, and nitrogenous materials (Lodder, 1934). Since there is such a close similarity between *T. lipofera* and the yeast isolated from New Jersey soils, cultures of *T. lipofera* might profitably be re-examined for spore formation.

The yeast might be classified under the family Endomycetaceae, subfamily

Saccharomycoidae, and tribe Saccharomycetaceae, but it cannot be classified as to the genus with certainty.³ In fact, it shows many characteristics which suggest closer agreement with some of the asporogenous yeasts than with the well-characterized genera of sporeforming yeasts.

On the basis of preliminary observations, Wickerham (1944) concluded that it was unlikely that the yeast belonged in any of the present genera of sporogenous yeasts since none of the latter contain yeasts with its characteristics, such as mucoid cell material, colored ascospores, ascospores in excess of eight when cultured on certain media, and a strong tendency of old cells to shed their outer membranes. In some respects the yeast resembles a culture described as *Debaryomyces hominis* by Todd and Herrmann (1936). However, the structures which were described as spores of *D. hominis* are different from the spores of the soil yeast, but comparative studies might show more similarity between the two than is apparent from the description of Todd and Herrmann.

Additional information is needed before the yeast can be completely classified and before it is possible to determine with certainty whether it is identical with any yeast which has already been described or is a new species.

Efficiency of conversion of carbohydrate to lipid. The yeast was cultivated in volumes of medium varying from 500 to 7,000 ml, in order to determine the amount of lipid contained in the cells and the relative portion of the carbohydrate which was converted to lipid. The medium commonly used had the following composition: tap water, 1,000 ml; glucose, 15 g; yeast extract, 0.1 g; K_2HPO_4 , 0.8 g; KH_2PO_4 , 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; and $FeSO_4 \cdot 7H_2O$, 0.025 g. The carbohydrate was sterilized separately from the rest of the medium. Since preliminary tests indicated that growth was much greater in aerated media, a vigorous stream of sterile air was bubbled through the medium throughout the period of incubation. The same yeast culture (strain 72) was used in all these experiments.

Extraction of lipid from the cells. The yeast was cultivated in 500 ml of medium, and the cell material was harvested after 5 days' incubation. It had grown well and produced a heavy, cream-colored suspension of cells which contained large lipid globules. The cells were removed by centrifugation, and the white, creamy cell paste was dried in a current of air at 50 C. After most of the water was removed, the material was dried at 60 to 70 C. Determinations were made on the medium for residual carbohydrate and reaction, and a portion of the cell material was tested for ash content. The rest of the cell material was extracted with ethyl ether. Only a small amount of material was obtained in the extract. The dried cell material was then extracted with chloroform, which removed less material than the ether. Chloroform extraction was followed by extraction with a boiling mixture of equal parts of ethyl ether and ethanol. A

³ Dr. L. J. Wickerham of the Northern Regional Research Laboratory at Peoria, Illinois, and the late Dr. A. T. Henrici offered many helpful suggestions and aided materially in characterizing the yeast and clarifying its taxonomic status. They have verified many of the results which are herein reported and have testified to the difficulty of identifying the yeast with any of the known species.

small additional amount of extract was obtained. The total amount of material extracted by the three solvents was 11.0 per cent of the initial cell weight. After each extraction the cell material was treated with the fat stain, Sudan III, and examined microscopically. None of the solvents altered the appearance of the lipid globules, which stained as well with Sudan III after extraction as before being treated. In an attempt to release the lipid, the cell material was hydrolyzed with 2 per cent HCl at 100 C for 4 hours. The acid was then neutralized, and the mixture was extracted with ether. An amount of material equal to 42.3 per cent of the original substance was extracted. The extract was a light-amber-colored fatty substance having a low melting point. As shown by the data in table 1, 53.3 per cent of the original cell material was extracted by the

TABLE 1
*Recovery of lipid from yeast cells**

Reaction of medium	
Initial pH ..	7.0
Final pH ..	3.8
Glucose content	
Initial.	6.350 g
Final	0.110 g
Glucose utilized . . .	6.240 g
Yeast cells	1.828 g
Conversion of glucose to yeast cells	29.3%
Ash content of cells	3.1%
Extraction of lipid from cells:	
1. Initial ether extract	3.2%
2. Extracted with chloroform after treatment 1	0.6%
3. Extracted with boiling mixture of 50-50 ether and ethanol after treatment 2	7.2%
4. Extracted with ether after hydrolysis with hot 2% HCl following treatment 3	42.3%
Total material extracted.....	53.3%
Conversion of glucose to lipid.....	15.6%

* Incubation period 5 days; aerated medium containing 0.01% yeast extract and 1.5% glucose.

solvents. Most of the glucose had been consumed, and 15.6 per cent as much lipid was recovered as glucose used. The yield of cell material was 29.3 per cent of the weight of the glucose used. The reaction of the medium became distinctly acid and dropped from an initial pH of 7.0 to 3.8.

Smedley-MacLean (1922) also found that only a small portion of the lipid of yeast cells with which she worked could be obtained by direct ether extraction. Several times as much lipid was obtained after hydrolysis with HCl (boiling normal HCl for 2 hours). She also reported that similar results had been obtained by Naegeli and Loew in 1878. Most of the yeast lipid was believed to be bound up in the cells with sterol, protein, and carbohydrate and became liberated on acid hydrolysis; a smaller part of the lipid which existed as free fat could be extracted directly with ether.

Yeast growth and lipid production. One 5-liter and two 2-liter portions of medium similar to the above but containing 3 per cent glucose were inoculated with the yeast and aerated for 33 days before harvesting. At the time of harvest the cultures were composed of typical large spherical cells each containing a single large lipid globule. It was unexpected to find the cells so well preserved after such a long incubation period. The cells were separated from the 5-liter portion of medium by centrifugation. The other two cultures were extracted without

TABLE 2
*Lipid production in glucose, nitrogen-deficient medium**

	CULTURE A	CULTURE B	CULTURE C
Period of incubation, days.....	33	33	33
Volume of medium, ml.	5,000	2,000	2,000
Reaction, pH			
Initial	7.0	7.0	7.0
Final... ..	3.0	2.9	2.9
Glucose content			
Initial	127.0 g	50.8 g	50.8 g
Final.....	22.2 g	0.8 g	5.0 g
Glucose utilized.. ..	104.8 g	50.0 g	45.8 g
Yeast cells... ..	21.175 g		
Conversion of glucose to yeast cells.....	20.2%		
Lipid extracted			
From cells.....	12.658 g		
From medium.....	0.227 g		
Total.....	12.885 g	5.811 g	5.144 g
Conversion of glucose to lipid	12.3%	11.6%	11.2%
Lipid content of cells.....	60.9%	57.5%†	55.6%†

* Aerated medium contained 3% glucose and 0.01% yeast extract.

† Calculated on basis of conversion of glucose to cells with culture A.

first separating the cells from the medium. The procedures were similar to those used in the preceding experiment except that the cell material and media were hydrolyzed with 2 per cent HCl and then neutralized before extraction, and ether was the only solvent used. Since the cells were not all recovered from culture A by centrifuging, both the medium as well as the separated cells were hydrolyzed and extracted. The results are reported in table 2. Most of the carbohydrate had been consumed by all three cultures. The conversion of glucose to cell material by culture A was 20.2 per cent, which was somewhat lower than the conversion in the experiment reported above. The lipid content of the cells, 60.9

per cent, was very high. Since the cells were not harvested from cultures B and C, the actual lipid content of the cells could not be calculated. An approximation can be obtained by assuming the same conversion of glucose to cells as in culture A (20.2 per cent). On this assumption, the lipid contents of cultures B and C are 57.5 and 55.6 per cent, respectively—values close to that for culture A. The conversion of glucose to lipid was similar with all three cultures and varied from 11.2 to 12.3 per cent. The reaction of the media became distinctly acid

TABLE 3
*Lipid production by yeast**

	CULTURE A	CULTURE B	CULTURE C	CULTURE D
Period of incubation, days.	12	19	25	32
Volume of medium, ml.	5,000	7,000	5,000	7,000
Reaction, pH				
Initial....	7.0	7.0	7.0	7.0
Final....	4.0	3.2	3.0	3.1
Glucose content				
Initial.....	127.0 g	177.8 g	127.0 g	177.8 g
Final ..	63.3 g	64.9 g	20.7 g	44.7 g
Glucose utilized ..	63.7 g	112.9 g	106.3 g	133.1 g
Yeast cells.....	14.93 g	25.81 g	20.60 g	22.31 g
Conversion of glucose to yeast cells.....	23.4%	22.9%	19.4%	16.8%
Lipid extracted				
From cells...	8.54 g	14.33 g	12.45 g	13.94 g
From medium ..			2.07 g	3.15 g
Total.....	8.54 g	14.33 g	14.52 g	17.09 g
Conversion of glucose to lipid	13.4%	12.7%	13.7%	12.8%
Lipid content of cells ..	57.3%	55.5%	60.4%	62.5%

* Aerated medium containing 3% glucose and 0.02% yeast extract.

during yeast growth, changing from an initial pH of 7.0 to pH 2.9 to 3.0. It is of interest that the conversions were so high in view of the long period of incubation.

The results of an additional experiment are presented in table 3. The cultures were grown in a medium similar to that used previously but containing 3 per cent glucose and 0.02 per cent yeast extract. Each of two containers held 5 liters of medium and two others each held 7 liters. One culture was harvested at each of the following incubation periods: 12, 19, 25, and 32 days. Throughout the periods of incubation the media were aerated.

The yeast cells of all cultures were filled with lipid at the time of harvest. The

cultures harvested at the 25- and 32-day periods showed some signs of deterioration, however. Many cells were shedding their outer membranes, and there was considerable turbidity of the medium after centrifuging. This turbidity was probably due to disintegration of some of the cells. There was some turbidity of the centrifuged liquid from cultures A and B but more from C and still more from D. In order to recover the lipid as completely as possible, both the cell material and the centrifuged liquid of cultures C and D were extracted with ether after being hydrolyzed with HCl and then neutralized. The extract obtained from the centrifuged liquid was 14 per cent of the total obtained from culture C and 18 per cent of the total from culture D.

The results are similar to those of the other experiments: (1) the reaction became strongly acid during growth; (2) there was between 16.8 and 23.4 per cent as much cell material as glucose consumed, and the efficiency of conversion was greatest at the shortest incubation period and decreased as the period was increased; (3) the amount of lipid extracted was equal to from 55.5 to 62.5 per cent of the total weight of the cells; and (4) there was from 12.7 to 13.7 per cent as much lipid produced as glucose consumed.

The results as a whole indicate that the yeast is a relatively efficient converter of carbohydrate to lipid and that the yields compare favorably with those reported for other yeasts and filamentous fungi (Fink, Haehn, and Hoerbinger, 1937; Geffers, 1937; Heide, 1939; Pruess and Strong, 1933; Raaf, 1941-1942; Rippel, 1943; Ward, Lockwood, May, and Herrick, 1935). During the course of the studies some of the cultures made relatively slow growth. The factors affecting the rate of growth and the efficiency of the conversion of carbohydrate to lipid have not been examined completely, but it is probable that the rate of growth can be accelerated and the efficiency of conversion increased by modifying the medium and changing the cultural conditions. Among the factors which may be expected to influence the transformation are the size of the inoculum, the nature and concentration of the source of carbon and nitrogen, the rate of aeration, and neutralization of the acid produced during growth.

The influence of the nitrogen content of the medium was shown by an experiment in which one medium contained 0.01 per cent yeast extract as the only source of nitrogen and a second medium contained 0.05 per cent $(\text{NH}_4)_2\text{SO}_4$ in addition. The cultures were grown in 5-liter portions of medium which were aerated during incubation for 20 days at 28 C. The results reported in table 4 are similar to those obtained in the previously reported experiments.

There was marked increase in acidity, and the final reaction of the medium containing ammonium sulfate was pH 2.6. The yeast extract medium contained inadequate nitrogen, as indicated by the fact that only 54 per cent of the carbohydrate was utilized; 89 per cent of the carbohydrate disappeared from the medium containing ammonium sulfate. Of particular interest is the efficiency of conversion of carbohydrate to cell material and lipid. The conversion to cell substance was much more efficient in the ammonium sulfate medium but the lipid content of the cells was much higher in the nitrogen-deficient medium, in which case the cells had the remarkably high lipid content of 65 per cent. The

cells grown in the ammonium sulfate medium contained 48 per cent lipid. The actual percentage of conversion of glucose to lipid was similar in both media, 10.3 per cent in the nitrogen-deficient medium and 11.6 per cent in the medium which contained ammonium sulfate, but there was much more total lipid in the latter medium.

The fact that under suitable cultural conditions there are rapid growth, nearly complete consumption of carbohydrate, and high-percentage conversion of carbohydrate to lipid suggests that the yeast might be cultivated for the production of fat from carbohydrate.

Kleinzeller (1944) made detailed studies of the conversion of carbohydrate to lipid by a similar and possibly identical yeast, *Torulopsis lipofera*. He reported

TABLE 4
*Influence of nitrogen content of medium on conversion of glucose to lipid**

	NITROGEN SOURCE	
	Yeast extract	Yeast extract and (NH ₄) ₂ SO ₄
Period of incubation, days.	20	20
Volume of medium	5,000	5,000
Reaction, pH		
Initial	7.0	7.0
Final	3.7	2.6
Glucose content		
Initial	148.5 g	148.5 g
Final	68.7 g	16.7 g
Glucose utilized	79.8 g	131.8 g
Yeast cells	12.55 g	31.58 g
Conversion of glucose to yeast cells.	15.7%	24.0%
Lipid extracted from cells	8.19 g	15.24 g
Conversion of glucose to lipid.	10.3%	11.6%
Lipid content of cells	65.3%	48.3%

* Aerated medium containing 3% glucose and 0.01% yeast extract.

that under favorable conditions the yeast converted 42 to 48 per cent of the carbohydrate to yeast cells (dry weight) which contained 18.6 to 43 per cent lipid. Fat formation was highest at pH 5.5 to 6.0 and at 20 to 25 C. The high nitrogen content of the medium depressed fat formation.

Nature of the yeast lipid. No tests have been made of the chemical composition of the lipid. The mixture as a whole had a low melting point. It persisted as a soft solid at 20 C and melted slowly as the temperature was raised until most was molten at 28 C; there were still some suspended solid particles at 35 C. Upon cooling there was no solidification at 18 C, but most of the lipid was solid at 15 C. When heated again there was little evidence of melting until the temperature reached 25 to 30 C.

The lipid of *T. lipofera* was characterized by Kleinzeller (1944) as follows: carbon content of fatty acids 76 per cent; unsaponifiable material 6.8 to 8.8 per

cent of the lipid; mean molecular weight of the fatty acids, 268 to 280; and iodine value 55 to 90.

The lipids obtained from other yeasts have been found to contain fats, free fatty acids, phospholipids, and sterols (Bloor, 1943; Prescott and Dunn, 1940; Anderson, 1939). Daubney and Smedley-MacLean (1927) reported that the principal fatty acids in yeast lipid were the saturated palmitic and the unsaturated oleic and linoleic acids. There were also small amounts of the saturated lauric acid and possibly some arachidic acid. According to Anderson (1939), yeast lipid contains such saturated fatty acids as palmitic, stearic, lauric, butyric, arachidic, and tetracosanoic acids, and also unsaturated C_{16} and C_{18} acids. Palmitic and stearic acids were the principal fatty acids. Yeast lipid is characterized by a high proportion of unsaponifiable matter comprising one-third or more of the material extracted (Smedley-MacLean and Thomas, 1920). About one-half of the unsaponifiable material was sterol in combination with fatty acids. The sterol separated out from the rest of the lipid on standing (Smedley-MacLean, 1922). The phospholipids of *Saccharomyces cerevisiae* have been reported to comprise more than one-half the total ether-soluble material (Anderson, 1939).

The lipid extracted from *Penicillium javanicum* was reported by Ward, Lockwood, May, and Herrick (1935) to be similar to that obtained from higher plants and consisted of glycerides of palmitic, stearic, tetracosanoic, oleic, and linoleic acids, with a small amount of unsaponifiable matter. Bernhauer and Posselt (1937) found that the lipid obtained from *Aspergillus niger* contained 6 per cent glycerol and 68 per cent total fatty acids, three-fourths of which were made up of unsaturated acids.

SUMMARY

The yeast was recovered from various soils and appears to be a common soil inhabitant. On plates of nitrogen-free, glucose agar seeded with soil and incubated for two weeks or more it produced large, spreading, slightly opaque colonies with the cells dispersed in a watery slime. On nitrogen-deficient media the cells were large and nearly spherical with no tendency to produce chains; most cells had only one small bud. Each cell contained a single large refractive fat globule, which nearly filled the cell. Old cells tended to shed their outer membranes. Spores were produced, but the number in each ascus was variable. The spores had a light amber color. Some strains produced up to 8 spores, but one strain produced 16 or more when cultivated on some media. Under certain conditions the spores were formed in a saclike bud which remained attached to the parent cell. The bud was at first filled with granular contents, which later changed to spores. No spores were formed in the parent cell. The metabolism of the yeast was oxidative and sugars were not fermented. Nitrate was not utilized and gelatin was not attacked. In solution media, growth was sedimentary with no pellicle formation, and relatively few cells remained suspended except in aerated media.

The characteristics of the yeast are different from those of previously described yeasts which have been studied for lipid synthesis. It was not possible

to identify the yeast with known species. The yeast may be a new species or a yeast already described but with certain characteristics which have not been recognized up to this time.

Growth and lipid production were good in aerated glucose solutions containing small amounts of yeast extract as the nitrogen source. Under favorable conditions, 20 to 25 per cent of the consumed glucose was converted to yeast cells which contained 50 to 63 per cent lipid. From 10 to 14 per cent of the consumed glucose was recovered as lipid. The lipid content of the cells decreased as the nitrogen content of the medium increased. Only a small portion of the lipid could be extracted from the cells directly with fat solvents, but it became released by hydrolysis with dilute acid.

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STUDIES ON CELLULOSE FERMENTATION

II. AN ANAEROBIC CELLULOSE-DECOMPOSING ACTINOMYCETE, *MICROMONOSPORA PROPIONICI*, N. SP.

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During an investigation¹ of cellulose decomposition by termites, anaerobic shake tubes containing cellulose, proteose-peptone, and agar were inoculated with serial dilutions of the crushed alimentary tract of a worker termite (*Am-termes minutus*) from a laboratory colony. Several large clear spots which appeared in the cellulose after several weeks were found to contain numerous minute branching filaments, suggesting that the causal organism was an actinomyce-ete. Since it appeared to differ from other cellulose-decomposing actinomyce-etes, some additional experiments were performed.

It was isolated in pure culture by inoculating it into shake tubes of cellulose or glucose with serial dilution and by subculturing similar series from a colony in the tube of highest dilution. A sparse formation of spores was observed in cultures grown on cellulose and proteose-peptone, but, if powdered dried grass or its aqueous extract was used in place of the proteose-peptone, an abundance of spherical spores developed. Their diameter averaged about $0.8\ \mu$. They were borne singly on short side branches of the mycelium (figure 1) in a manner characteristic of the genus *Micromonospora*.²

The oxygen relationships of this strain of *Micromonospora* were studied by inoculating parallel series of aerobic and anaerobic dilution tubes containing cellulose, agar, and grass extract.

In the anaerobic series all air was displaced by bubbling oxygen-free nitrogen (95 per cent) and carbon dioxide (5 per cent) through the test tube before stoppering. In the aerobic series alveolar air (approximately 15 per cent O_2 , 5 per cent CO_2 , and 80 per cent N_2) was similarly bubbled. The tubes were then stoppered with a sterile rubber stopper, inverted several times, and quickly cooled in cold water to solidify the agar. This left a thin film of the agar medium lining the wall in the upper, gas-filled half of the tube. After two weeks of incubation cellulose digestion was evident in all parts of the anaerobic tubes, including the thin layer of cellulose agar in the upper half. In the aerobic series no colonies appeared in this thin agar layer exposed to the gas, nor were any colonies present in the upper 3 cm of solid agar in the lower part of the tubes. Colonies

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²The author is indebted to Dr. A. R. Colmer for the identification of this organism as a member of the genus *Micromonospora*, and also for suggestions on the manuscript.

appeared only in the depths of the aerobic tubes. These results indicate the anaerobic nature of the organism.

Growth is extremely slow in all media tested, two to four weeks being required for the development of visible colonies. This agrees with the slow growth of the aerobic forms isolated by Colmer and McCoy (1943). Temperatures of 30 to 40 C gave the most rapid development.

Glucose and cellulose are both suitable as a source of carbohydrate. Other sugars have not been tested. In addition to the carbohydrate, complex organic materials must be present. Extracts of liver, yeast, and dried grass support relatively rapid growth and these cannot be replaced by a mixture of pantothenic acid, thiamine, nicotinic acid, riboflavin, pyridoxine, biotin, and folic acid.



FIG. 1. PORTION OF A YOUNG COLONY OF MICROMONOSPORA SHOWING METHOD OF SPORE FORMATION (GRAM STAIN)

Colonies in cellulose agar are first visible as tiny clear areas in which the cellulose has been digested. As the size of the clear area increases, a white colony can be seen in the center. If deeply imbedded in cellulose, the colony enlarges equally in all directions, maintaining a spherical shape and completely digesting all cellulose in the region which it occupies. Older colonies differ macroscopically only in size from younger ones. If a colony develops adjacent to the glass, a difference in the interior of young and old colonies can usually be detected. Whereas colonies 1 mm or less in diameter display a uniform white opacity, the larger ones show this only in the outer part. The center is relatively transparent. Continued growth consists of an expansion of the outer white shell, which remains about 0.5 mm thick, with a corresponding enlargement of the central transparent region.

When a colony grows in the thin layer of agar lining the gas-filled part of the tube, it appears to consist of a white ring which gradually increases in diameter. A culture tube showing this is illustrated in figure 2.

If a thin section is prepared of an older colony imbedded in agar, it is found that it also consists of a white outer shell and a transparent center. When the section is examined microscopically, the extreme periphery of the colony is found to be composed of vegetative filaments which extend radially toward the undigested cellulose. They are separated from it, however, by a thin layer in which the cellulose is digested, but which contains no filaments, indicating that an extracellular cellulase is secreted. Inside the peripheral layer there is another layer or shell in which numerous spores are produced. Because of the scattering of light by the spores this is the part of the colony which shows the white opacity. The central transparent portion is relatively devoid of spores. Filaments are scarce, and in fresh mounts they are very indistinct. They fail to take the gram

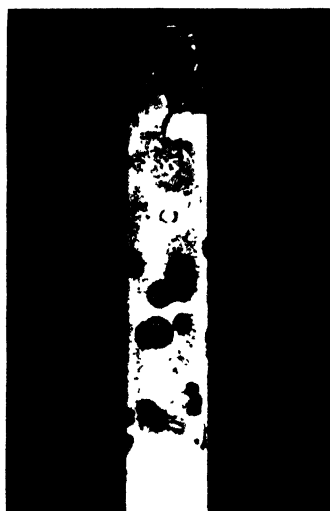


FIG. 2. COLONIES OF *MICROMONOSPORA* GROWING IN THIN CELLULOSE AGAR, ILLUSTRATING THE RINGLIKE POSITION OF THE SPORES

stain, in contrast to the young vigorous filaments on the periphery. The growing colony thus consists of a gradually expanding hollow shell, its outer surface composed of vegetative filaments, the adjacent inner portion containing numerous spores, and the center relatively devoid of protoplasm.

Two explanations for the disappearance of the spores and filaments in the central portion of the old colony may be suggested. They may be killed by the accumulation of metabolites and then undergo autolysis, or they may be resorbed and their substance transported to the peripheral portion where it is used in the synthesis of new cells. It has been noted that the outer, white shell of spores was still present in old tubes 3 years after they were inoculated and long after growth had ceased. These tubes contained maximal amounts of metabolic products. If accumulation of metabolites caused the disintegration of the central part of the growing colony, it would be expected that it would also cause the spores in the old tubes to disappear. This does not occur. Furthermore, in a growing colony the outer filaments and spores constitute the most

active protoplasm. Consequently metabolic products would be as concentrated there as in any other region, including the center, yet the spores and filaments remain active. This shows that accumulation of metabolites cannot account for the absence of protoplasm in the center of the colony. It may be postulated that the various parts remain integrally related and that as materials are needed for growth in the peripheral portions they are drawn in part from the central region. A phenomenon of this sort is common in many of the higher fungi but is not usually encountered among lower forms.

On glucose the organism forms a solid rather than a hollow shell type of colony. The solubility of glucose and the insolubility of cellulose suggest an explanation for this difference. Glucose readily diffuses to the cells, and there is little stimulus to outward growth. On the other hand, digestion of cellulose adjacent to the colony increases the distance between the organism and the substrate, thereby reducing the efficiency with which the remaining cellulose may be utilized. Transfer of the protoplasm from the center to the periphery of the colony decreases its distance from the substrate and leads to more rapid utilization.

TABLE 1
Fermentation balances for Micromonospora

SUBSTRATE	PRODUCTS IN MILLIMOLES		
	CO ₂	Acetic acid	Propionic acid
80 mg glucose	0.275	0.280	0.392
100 mg glucose	0.259	0.325	0.455
586 mg cellulose		0.87	1.73

Several cultures started with known amounts of glucose or cellulose have been analyzed for fermentation products, using methods already indicated (Hungate, 1944). Carbon dioxide and acids are produced. No hydrogen or neutral volatile products are formed. In one experiment the total acid produced (exclusive of CO₂) was found to be 0.794 milliequivalents. The volatile acid was 0.78 milliequivalents, indicating that the major part of the acids produced was volatile.

The Duclaux distillation of the volatile acid gave values intermediate between those for acetic acid and propionic acid. No formic acid was present. Acetic acid was identified as the sodium uranyl salt. In order to determine the nature of the other acids a fractional precipitation with silver nitrate was performed, and the silver contents of the first three fractions were determined. These were found to be 59.5, 58.6, and 58.5 per cent, respectively. This indicates the presence of propionic acid, silver content 59.63 per cent. No significant amounts of higher fatty acids were present.

From the values for the Duclaux distillation the relative amounts of acetic and propionic acids were determined by reference to the figures recorded by van Niel (1928). In several experiments the ratio of propionic to acetic acid was found to vary between 1.4 and 2.0. Fermentation data for three cultures are shown in table 1.

The fermentation products account for about 70 per cent of the carbon in the substrate. The amounts in which CO_2 , acetic, and propionic acids are recovered indicate that the remaining materials have approximately the formula of a carbohydrate. The proportion of unidentified products is similar to that observed in the fermentation of glucose and cellulose by *Clostridium cellobioparus* (Hungate, 1944).

The ratios in which carbon dioxide, acetic, and propionic acids occur in the fermentation by *Micromonospora* are those commonly encountered in fermentations by the propionic acid bacteria (van Niel, 1928). Because of this feature it is appropriate to designate the present organism as *Micromonospora propionici*, n. sp. It is distinguished from other species of *Micromonospora* by its obligate anaerobic nature and its characteristic fermentation products. No colored pigment is formed.

In an old culture which was supplied with more cellulose than could be fermented, the extra cellulose disappeared after prolonged incubation. The culture medium showed significant copper reduction with Benedict's solution. Glucose was demonstrated as the phenyllosazone. No indications of cellobiose were observed and the reducing power of the culture was not increased by acid hydrolysis, indicating that the reduction was due to simple sugars. It is probable that glucose is the chief product of cellulose digestion by this organism. This demonstration of a cellulase in an old culture of *Micromonospora* is in agreement with the observation that an area of digested cellulose surrounds colonies growing in cellulose agar.

The significance of *M. propionici* to the termite from which it was isolated is of interest because in many wood-eating termites cellulose digestion depends on symbiotic microorganisms. The number of colonies developing in the original dilution series inoculated with the alimentary tract of a single *Amilermes* worker indicated that about 500 colony-producing units of *Micromonospora* were present in the gut. Although its occurrence in this number suggests that it may have been of some significance in the carbohydrate nutrition of the host, it does not appear probable that it is of major importance in this respect. Microscopic examination of a smear prepared directly from the gut of one of the termites failed to disclose any structures which could be identified as *Micromonospora*. The slow growth of *Micromonospora* in laboratory cultures also suggests that it would be of limited utility in the symbiotic digestion of cellulose.

M. propionici is not restricted to the termite gut. An anaerobic strain of *Micromonospora* indetical in appearance with it has been encountered and isolated from a culture of the protozoa from the rumen of cattle (Hungate, 1942). R. Meyer (1934) pictures a cellulose-digesting actinomycete which he observed in one of his anaerobic cultures. The morphology of the sporulating filaments shown in his photographs appears similar to that for *M. propionici*. The green color and the capacity for aerobic growth which he reported show that a different species was concerned.

DISCUSSION

An actinomycete exhibiting a characteristic propionic fermentation is of interest from the standpoint of the evolution of the group. It has been postulated

on morphological grounds that the actinomycetes originated from the propionic acid bacteria (Stanier and van Niel, 1941). The latter show a tendency toward branching which finds a greater expression in the actinomycetes. *Micromonospora*, as one of the more primitive actinomycetes, might be expected to have a closer relationship to the propionic acid bacteria. The demonstration that it carries on a propionic acid fermentation provides physiological evidence supporting the hypothesis of an origin of the actinomycetes from the propionic acid bacteria.

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PENICILLIN

VIII. PRODUCTION OF PENICILLIN IN SURFACE CULTURES¹

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The production of penicillin by the surface culture of *Penicillium notatum* is the foundation upon which a large new industry has been built. Although cultivation of the mold in submerged culture now appears to be more economical and more practical from an industrial standpoint, surface cultures were utilized to produce the penicillin that first effected the remarkable clinical cures which indicated that the large investment of time and money made during the past three years would be justified.

Previous investigators of penicillin production have not been successful in developing a highly productive medium. Fleming (1929), the discoverer of the drug, refers only to the use of a "nutrient broth," whereas Clutterbuck, Lovell, and Raistrick (1932) used a modified Czapek-Dox medium. Although the yields of penicillin obtained by this group and by Fleming were not evaluated in terms of the Oxford unit adopted later, they were undoubtedly very low, at least when compared with present standards. Abraham *et al.* (1941), using the same modified Czapek-Dox medium with the addition of small amounts of a crude yeast extract, obtained only 2 to 6 Oxford units per ml.

This paper will report on a few of more than five hundred experiments which have led to an increase in the yield of penicillin from the range of 2 to 6 Oxford units per ml to as much as 160 to 220 Oxford units per ml. This increase in yield has been achieved primarily by the proper selection of organisms and nutrients, including the use of corn steep liquor, the use of lactose as the principal carbohydrate, and the addition of nutrients during the course of the fermentation.³

METHODS AND MATERIALS

All of the assays were conducted by the cylinder-plate method (Abraham *et al.*, 1941), as modified by Schmidt and Moyer (1944). The method has the advantage that it gives no response to notatin (penatin, penicillin B) and there-

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³Since March, 1942, the results of this work have been distributed in accordance with government regulations by the Committee on Medical Research to penicillin producers and many research groups in this country and abroad. Owing to the strategic significance of penicillin, it was considered advisable to delay publication of these results.

fore gives a true measure of penicillin. This fact has frequently been confirmed in this laboratory by extraction into ether of the penicillin from high-yielding cultures, followed by transfer to a buffer and reassay. However, it must be borne in mind that the results obtained by the cylinder-plate method may be in error by as much as approximately 10 per cent because of the inherent limitations of the procedure.

The production cultures were grown in 200-ml pyrex Erlenmeyer flasks containing 50 ml of the nutrient medium. Inoculations were made with a generous application of dry, ungerminated spores. In each experiment a sufficient number of flasks were employed so that duplicate cultures could be harvested and assayed on several consecutive days. All production cultures were incubated at $24\text{ C} \pm 1$.

A slightly modified Czapek-Dox solution contained, as the standard salt constituents, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.250 g; KH_2PO_4 , 0.500 g; NaNO_3 , 3.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.044 g; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004 g per liter of final medium. This standard medium was supplemented with various carbohydrates and nitrogenous materials as desired.

Various lots of concentrated corn steep liquor,⁴ typical of the commercially available product, were employed. This material is known to vary considerably in composition; however, a typical analysis is as follows: total solids, 52 per cent; total nitrogen, 4.3 per cent; ash, 7.9 per cent; free reducing sugar, 5.6 per cent; total reducing sugar calculated as glucose after acid hydrolysis, 6.8 per cent; specific gravity, 1.25; and pH, 4.0.

EXPERIMENTAL RESULTS

Organism Selections

In the first experimental work use was made of a strain of *Penicillium notatum* Westling which was supplied by Dr. H. W. Florey of Oxford University, and which was descended from the original Fleming organism. This strain was not stable with respect to sporulation or penicillin production; therefore, very early in the research program, a search was made for a superior organism.

This survey of species of *Penicillium* extended over a considerable period, during which time improvements were being made in the culture medium. About 35 strains of *P. notatum* and *Penicillium chrysogenum* were investigated, including many cultures previously obtained for the culture collection of this laboratory (largely from the Thom collection), as well as several descendants of the original Fleming strain which were kindly placed at our disposal by other

⁴Corn steep liquor is a by-product of the starch industry. Before the corn is ground, it is steeped for approximately 30 hours in water originally containing 0.1 to 0.3 per cent of sulfur dioxide. The water has previously washed the starch and passed through the gluten settling tank. The addition of sulfur dioxide, at the time steeping begins, inhibits fermentation. Prior to concentration, a lactic acid fermentation occurs to a variable extent. There is considerable variation in sugar and lactic acid contents. Steep liquor is sold on a basis of approximately 55 per cent solids, although different batches may vary rather widely from this value.

investigators. Some of the *P. chrysogenum* strains gave promising yields of penicillin but were not superior to some of the descendants of the original Fleming organism. The strain finally selected for further nutritional investigation was obtained through the generosity of Dr. George Harrop, of the Squibb Institute of Medical Research. This strain, now known as NRRL 1249, was a descendant

TABLE 1
Comparison of penicillin yields from several strains of penicillium

ORGANISMS*	CULTURE AGE—DAYS			
	4	5	6	7
Penicillin yield—units per ml				
NRRL				
831.....	34	45	59	69
832.....	37	48	63	69
833.....	20	30	36	39
828.....	4	5	13	19
815.....	17	29	49	58
1249—type A.....	36	55	68	76
1249.B21.....	65	91	138	147
pH of filtrates				
831.....	6.2		6.7	7.6
832.....	6.6		7.2	7.5
833.....	5.7		7.1	7.6
828.....	5.8		6.6	6.8
815.....	6.4		6.8	7.1
1249—type A.....	5.5		7.2	7.7
1249.B21.....	5.7		7.2	7.6
Mycelium weight—g per culture				
831.....	0.88		1.11	1.10
832.....	0.79		1.02	1.00
833.....	0.91		1.20	1.23
828.....	0.68		1.03	1.34
815.....	0.79		1.06	1.00
1249—type A.....	0.78		1.11	1.23
1249.B21.....	0.76		1.05	1.08

Culture medium: 10 g concentrated corn steep liquor, 4.0 g lactose per 100 ml, and standard salts.

* NRRL 831, 832, 833, and 828 are cultures of *P. notatum* obtained from the Thom collection as B-47A, B-69, B-464, and 5646II, respectively. They are not descended from the Fleming strain. NRRL 815 came from the Thom Collection as B-508 and is listed as *P. chrysogenum*. NRRL 1249 type A and 1249.B21 arose in this laboratory as variants from NRRL 1249 and are descended from the Fleming strain of *P. notatum*.

of the Fleming strain and was chosen because it was one of the best for penicillin production and produced a more luxuriant crop of spores than many of the other strains.

Data from a portion of this survey, obtained with an improved culture medium, are presented in table 1. It is evident that there was a great variation in the

capacity of the various organisms to produce penicillin. Most of the strains had essentially the same growth rate. The amount of yellow pigment accumulating in the liquid medium varied from strain to strain, although there appeared to be no correlation between penicillin yield and pigment formation. This survey failed to reveal an organism which was superior to some of the descendants of the original Fleming strain.

Spore Production and Inoculation

A large number of nutrient combinations were employed in an effort to devise a medium upon which a thin, heavily sporulating mycelium would develop. Good results were obtained with the following combination, which is suitable for use as either a liquid or jellied medium:

Sporulation medium

Glycerol.....	7.5 g
Cane molasses (edible quality as commonly sold at retail).....	7.5 g
Corn steep liquor.....	2.5 g
MgSO ₄ ·7H ₂ O.....	0.050 g
KH ₂ PO ₄	0.060 g
Peptone.....	5.00 g
NaCl.....	4.00 g
Fe-tartrate.....	0.005 g
CuSO ₄ ·5H ₂ O.....	0.004 g
Agar.....	2.50 g
Distilled water to make 1 L	

When this medium is inoculated with a spore suspension and shaken vigorously prior to incubation, a very uniform surface growth results. By the addition of increased quantities of agar (about 2.5 per cent), the medium can be made to jelly for use in test tube slants or petri dish cultures.

By using an alternative method, many times the number of spores can be produced per flask. Fresh whole-wheat bread, which must not contain any commercial mold inhibitor such as "mycoban," is cut into 1-cm cubes and steam-sterilized in shallow layers in Erlenmeyer flasks. It is then heavily inoculated with spores. In 4 to 5 days, at 25 to 27C, a heavy crop of spores develops inside and outside the bread. When the spores have been formed and there has been some drying of the spore-covered pieces of bread, these cultures can be stored at 4C for at least 2 weeks without apparent loss in vitality or ability to produce penicillin. After 6 or 7 days, the bread crumbles and is easily reduced to a powdery mass, which may be used directly for inoculation or may be blended with 3 to 4 volumes of a mixture of equal parts of whole-wheat flour and finely ground oat hulls, which will float and spread rapidly over the surface of the medium. Portions of such a spore-bearing mixture can be conveniently introduced into the sterile medium by means of a spatula or atomizer. Inoculations performed in this manner result in rapid and uniform surface growth. These improvements have resulted in greater uniformity among the production cultures and a considerable decrease in the time required to make the inoculation.

Variant Strains and Culture Maintenance

Very poor sporulation was sometimes encountered in liquid cultures inoculated with spores of *P. notatum* NRRL 1249 which had been grown for several generations on the agar sporulation medium. These poorly sporulating cultures were characterized by the appearance of white aerial growth, which was either confined to small spots or generally distributed over the surface of the mycelium. Such cultures were discarded, and only the best sporulating cultures were used to inoculate production flasks.

This method of culture selection was found to be inadequate as a means of insuring uniformity in the appearance of the culture and in the yield of penicillin. When sporulating cultures so selected were used to inoculate a production medium containing lactose and corn steep liquor, the resulting mycelium occasionally showed varying numbers of small white spots; in some cases the whole mycelium exhibited practically no sporulation. Such nonsporulating cultures gave low yields of penicillin (50 to 60 units per ml), as contrasted with 100 to 120 units per ml from other production cultures having a good crop of spores. This apparent correlation between culture appearance and penicillin yield was believed to be due either to some unknown variable in the nutrient medium or to the presence of two or more strains in the stock culture. Monospore cultures were accordingly prepared from one of the stock cultures which had been grown for several generations on a good agar sporulation medium. When these monospore selections were cultivated on a lactose corn steep liquor production medium, two types of mycelia were observed: type A produced practically no spores and only 50 to 60 units of penicillin per ml, whereas type B produced a good crop of spores and penicillin yields of 100 to 120 units per ml (table 1). Type B colonies closely resembled the parent strain, NRRL 1249. One of the type B strains was again subjected to monospore selection; all mycelial growth resulting from the selection sporulated well and gave good yields of penicillin. One of these monospore selections, *P. notatum* NRRL 1249.B21 (isolated and evaluated by the senior author), has been used in most of the early investigations conducted at this laboratory and because of its desirable characteristics is today generally used throughout industry for the production of penicillin in surface cultures. The superiority of this strain has been substantiated by later surveys conducted by Raper *et al.* (1944) of this laboratory.

In order to maintain the potency of this organism and to minimize the appearance of inferior mutant strains, it is recommended that spores be maintained in lyophil or dry soil tubes and that subcultures be made from such preparations at frequent intervals.

Optimum Conditions for Penicillin Production

Medium. The culture medium recommended by Dr. H. W. Florey and Dr. N. G. Heatley (personal communication on July 16, 1941) was the Czapek-Dox medium, supplemented with 4 per cent of glucose and 5 to 10 per cent by volume of a crude yeast extract. Two to six units of penicillin per ml could be obtained

on this medium in 7 to 8 days. Improvements have gradually been made in this culture medium, with the result that 150 to 200 units per ml now can be obtained in 5 to 6 days. The compositions of the original medium and of the medium considered near optimum today are as follows:

	ORIGINAL MEDIUM	IMPROVED MEDIUM
NaNO ₃	3.0 g	3.0 g
MgSO ₄ ·7H ₂ O	0.500 g	0.250 g
KH ₂ PO ₄	1.00 g	0.500 g
KCl	0.50 g	None
FeSO ₄ ·7H ₂ O	0.010 g	None
Glucose monohydrate	40.000 g	2.75 g
ZnSO ₄ ·7H ₂ O	None	0.044 g
MnSO ₄ ·4H ₂ O	None	0.004 g
Lactose monohydrate	None	44.0 g
Corn steep liquor	None	100. g
Crude yeast extract	50-100 ml	None
Initial pH	4.6	4.6
Water to make one liter		

The composition of the improved medium was determined after many experiments and will be discussed under various headings as given below.

Carbon sources. When the Czapek-Dox medium (without corn steep liquor) was used, there was no marked difference in the response to various carbon sources, as indicated by penicillin production, except that lactose was inferior because it supported only a trace of fungus growth. However, once the effectiveness of corn steep liquor was recognized and this material was added to the basic medium, a marked effect of various carbon sources on penicillin yield was observed.

The results obtained from a comparison of several common carbon sources, each of which was used in 3 per cent concentration in steep-liquor standard salt medium, are given in table 2. In the corn steep liquor there was sufficient assimilable carbohydrate, probably mainly glucose and dextrans, to support fairly good fungus growth and moderate penicillin production, even in the absence of added carbon sources. Lactose, cornstarch, and corn dextrin were equally good for penicillin production, glycerol being definitely inferior. No significant difference in penicillin production could be observed between cultures containing commercial glucose and those containing brown sugar. The pH of the broth did not rise so fast in the lactose, starch, and dextrin cultures as it did in the glucose, sucrose, and glycerol cultures.

A further comparison of these carbon sources was made at 6 per cent concentration (table 3). Again cultures containing lactose gave the highest penicillin yields. The change in the pH of the broth was slower in the cultures containing lactose than in those containing glucose, sucrose, glycerol, or sorbitol. Increasing the concentrations from 3 to 6 per cent under these conditions failed to increase the penicillin yields.

In these experiments, the cornstarch was added as a dry powder to the small culture flasks containing 50 ml of the basic medium. The starch was then

TABLE 2

Penicillin production from various carbon sources of 3 per cent concentration

CARBON SOURCE	CULTURE AGE—DAYS				
	3	4	5	6	7
	Penicillin yield—units per ml				
Control (no added carbon).....	27	45	41	36	27
Glucose.....	18	54	91	95	66
Brown sugar.....	7	40	85	102	79
Lactose.....	36	63	112	138	146
Glycerol.....	14	43	80	74	52
Cornstarch.....	35	85	122	140	146
Corn dextrin.....	28	73	91	125	146
CARBON SOURCE	pH of filtrates				
	3	4	5	6	7
	pH of filtrates				
Control (no added carbon).....	6.0	7.5	8.0	8.1	8.3
Glucose.....	4.6	6.1	7.3	8.1	8.2
Brown sugar.....	4.3	5.4	7.0	7.8	8.1
Lactose.....	4.7	5.7	6.7	7.4	7.8
Glycerol.....	4.8	6.2	7.5	8.1	8.2
Cornstarch.....	4.9	6.6	7.1	7.4	7.7
Corn dextrin.....	4.7	6.0	6.7	7.4	7.7

Culture medium: Carbon source, 3.0 g per 100 ml; corn steep liquor, 10 g per 100 ml; standard salts; and initial pH, 4.0.

Organism: *P. notatum* 1249.B21.

TABLE 3

Penicillin production from various carbon sources at 6 per cent concentration

CARBON SOURCE	CULTURE AGE—DAYS					
	6			8		
	Penicillin yield	pH of filtrates	Mycelium weight per culture	Penicillin yield	pH of filtrates	Mycelium weight per culture
	units per ml		g	units per ml		g
Control*.....	40	8.2	.51	10	8.4	.41
Lactose.....	105	7.2	1.14	133	7.8	1.15
Glycerol.....	40	8.1	1.31	14	8.4	1.13
Sorbitol.....	42	8.1	1.28	18	8.4	1.07
Brown sugar.....	45	7.5	1.48	58	8.3	1.32
Glucose.....	50	7.6	1.31	30	8.4	1.14

Culture medium: Carbon source, 6.0 g per 100 ml; corn steep liquor, 10 g per 100 ml; standard salts; and initial pH, 4.0.

Organism: *P. notatum* NRRL 1249.B21.

* No carbon source added.

gelatinized by heating and shaking the flasks in a water bath. Starch which was liquefied by an acid or malt treatment was easier to handle and gave good

penicillin yields. A commercial 80-fluidity starch, which was readily suspended in the culture medium by heating and stirring, appeared to be slightly better for penicillin production than untreated starch.

Since cornstarch was a satisfactory carbohydrate, tests were made to determine whether crude sources of starch could be used. Whole corn and whole wheat were ground to pass a $\frac{1}{8}$ -inch screen and were added to the basic medium in varying quantities. Ground corn, ground wheat, and granular wheat flour were as satisfactory as lactose for penicillin production (table 4). Although, under these conditions, ordinary starch and the crushed grains gave good penicillin yields in the small flasks, difficulty was encountered in obtaining an even distribution of the grain particles in large bottles, especially in tall bottles which had to be sterilized in an upright position and later placed horizontally.

To develop the idea of using crude carbohydrate sources further, a corn mash was prepared by mixing 150 g of ground, yellow corn with 1,500 ml of water,

TABLE 4
Penicillin production from medium containing ground corn and wheat

CARBON SOURCE		CULTURE AGE—DAYS				
Type	g/100 ml	4	5	6	7	8
Penicillin yield—units per ml.						
Ground corn.....	4.3	46	92	95	109	140
Ground corn.....	6.0	55	80	120	150	145
Ground wheat.....	4.5	46	98	110	127	140
Ground wheat.....	7.4	50	85	115	140	140
Granular wheat flour.....	3.7	53	90	112	138	119
Cornstarch.....	3.0	56	68	102	127	127
Lactose.....	3.0	64	85	109	143	147

Culture medium: Corn steep liquor, 10 g per 100 ml; standard salts; and initial pH, 4.0.
Organism: *P. notatum* 1249.B21.

gelatinizing the mixture in a hot-water bath, and then autoclaving it for 30 minutes at 15 lb to stop enzymatic activity. The resulting mash was fairly fluid and could be mixed readily with the basic medium. Portions of this mash were employed to provide 4 and 6 per cent starch concentrations in media for penicillin production. The penicillin yields compared favorably with those obtained with whole grain, dextrin, and unhydrolyzed starch (table 5).

Mixtures of lactose and ground corn were satisfactory for penicillin production. The results obtained when 1 per cent of lactose and 4 per cent of ground corn were employed in production media are shown in table 6. The slow utilization of the corn prevented a too rapid rise in the pH of the medium; the pH of the broth did not rise above 7.8, and there was no decrease in penicillin yield during the 9-day incubation period. Subsequent investigations showed that a slightly faster accumulation of penicillin was obtained when a mixture of 2 per cent of lactose and 2 per cent of ground corn was used.

Because of its solubility lactose is easier to handle in nutrient medium prepara-

tion than is starch or ground grain. By using the best culture conditions thus far developed, the effect of lactose concentration on penicillin production was determined (table 7). The highest penicillin yields were obtained in 5 days by using a lactose concentration of 4 per cent. The higher the lactose concentration, the slower the rise in the pH of the culture medium. The fungus growth was not significantly greater in the 5.5 and 7.0 per cent lactose cultures.

TABLE 5
Penicillin production from medium containing malted corn mash

STARCH CONTENT OF FINAL MEDIUM	CULTURE AGE—DAYS				
	4	5	6	7	8
	Penicillin yield—units per ml				
per cent					
4	41	112	122	132	110
6	26	82	117	146	129

Culture medium: Corn steep liquor, 8.75 g per 100 ml; standard salts; and initial pH, 4.2.
Organism: *P. notatum* 1249.B21.

TABLE 6
Penicillin production from medium containing ground corn and lactose

CARBOHYDRATE SOURCE	CULTURE AGE—DAYS					
	4	5	6	7	8	9
	Penicillin yield—units per ml					
1% lactose.....	55	62	59	55	60	45
1% lactose + 4% corn.....	44	68	111	138	157	159
	pH of filtrates					
	6.6	7.4	7.8	8.0	8.15	8.25
	5.4	6.2	6.9	7.5	7.8	7.8
	Mycelium weight—g per culture					
	0.54	0.60	0.62	0.58	0.54	0.48
	0.66	0.82	0.91	0.90	0.88	0.86

Culture medium: Corn steep liquor, 10 g per 100 ml; standard salts; and initial pH, 4.0.
Organism: *P. notatum* 1249.B21.

Some experiments were made to determine the ability of the fungus to utilize lactic acid or salts of lactic acid which were known to be present in the corn steep liquor. Various concentrations of lactic acid were used in media low in glucose and corn steep liquor. Both *P. notatum* NRRL 1249.B21 and NRRL 832 produced more mycelial growth on media to which the lactic acid had been added (table 8). Under similar conditions calcium lactate gave increased mycelial growth. Direct determinations of the lactic acid were not made but the rise in pH and the increase in fungus growth were considered as evidence that lactic acid or its salts served as carbon sources for these organisms.

TABLE 7

Effect of lactose concentration on penicillin production

LACTOSE CONCENTRATION	CULTURE AGE—DAYS					
	3	4	5	6	7	8
Penicillin yield—units per ml						
<i>g per 100 ml</i>						
1.0	42	43	34	18	3	1
2.5	53	80	80	68	39	18
4.0	53	112	192	188	168	149
5.5	71	112	180	188	184	146
7.0	66	100	160	188	178	134
pH of filtrates						
1.0	7.2	8.05	8.2	8.4	8.7	8.8
2.5	6.9	7.55	8.0	8.3	8.4	8.7
4.0	6.5	6.9	7.5	7.8	8.2	8.4
5.5	6.4	7.0	7.4	7.8	8.2	8.35
7.0	6.2	6.9	7.2	7.5	7.8	8.2
Mycelium weight—g per culture						
1.0	0.65	0.72	0.69	0.56	0.51	0.50
2.5	0.74	0.94	1.01	0.91	0.81	0.75
4.0	0.81	1.05	1.26	1.24	1.13	0.99
5.5	0.91	1.17	1.37	1.34	1.30	1.18
7.0	0.88	1.11	1.30	1.50	1.43	1.32

Culture medium: Corn steep liquor, 10 g per 100 ml; glucose, 0.275 g per 100 ml; standard salts; media adjusted with KOH to initial pH, 4.6.

Organism: *P. notatum* 1249.B21.

TABLE 8

Growth and pH change in media containing various concentrations of lactic acid

LACTIC ACID ADDED PER LITER	INITIAL pH	CULTURE HARVESTED AT 5 DAYS			
		<i>P. notatum</i> NRRL 832		<i>P. notatum</i> NRRL 1249.B21	
		pH of filtrates	Mycelium wt per culture	pH of filtrates	Mycelium wt per culture
<i>g</i>			<i>g</i>		<i>g</i>
0	3.9	8.0	0.60	7.8	0.54
3.6	3.3	8.15	0.64	7.8	0.59
5.4	3.2	8.0	0.71	7.9	0.60
7.2	3.1	7.95	0.74	7.75	0.64
9.0	3.0	7.8	0.75	7.6	0.66
10.8	2.95	7.6	0.78	7.3	0.67
12.6	2.9	7.6	0.83	7.2	0.71
14.4	2.85	7.3	0.84	6.9	0.76
16.2	2.8	6.85	0.83	5.2	0.77
18.0	2.75	6.1	0.81	4.55	0.65

Culture medium: Corn steep liquor, 2.5 g per 100 ml; glucose, 2.0 g per 100 ml; and standard salts.

* A comparison of penicillin production, carbohydrate utilization, pH change, and growth rate on a glucose and on a lactose medium (standard salts plus 10 per cent corn steep liquor) is shown in figure 1. The maximum penicillin yields of 143 and 84 units per ml from lactose and glucose media, respectively, were attained between the fifth and sixth days, after which there was a decrease in the penicillin content of the broth. The total reducing power of the medium was determined after acid hydrolysis and was calculated as glucose. The cultures on the glucose medium grew slightly faster than those on the lactose medium, which corresponds with the observation that the glucose was consumed more

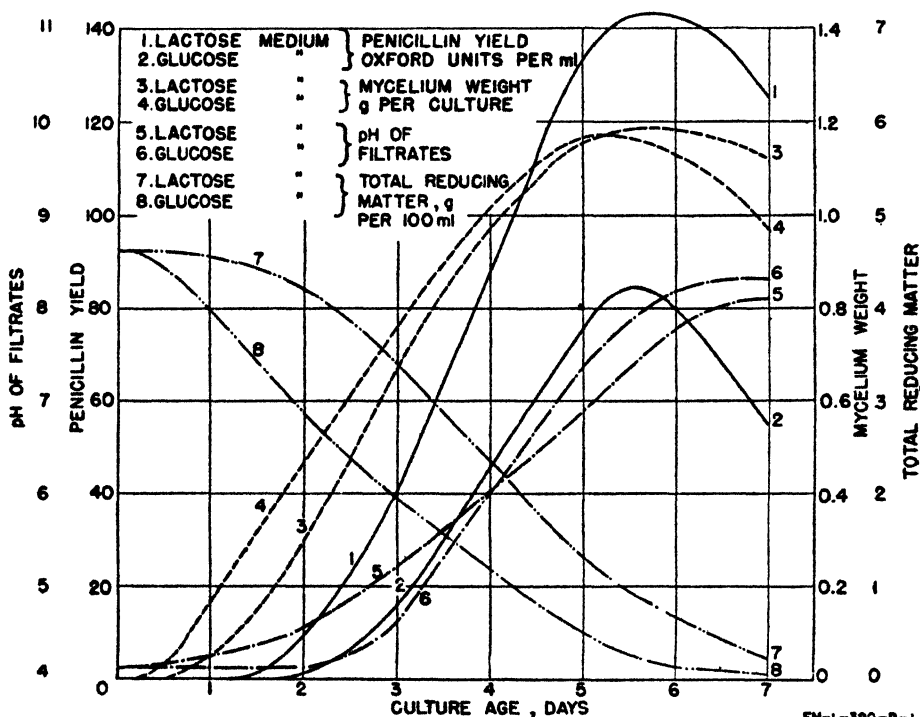


FIG. 1. PENICILLIN PRODUCTION, GROWTH, pH CHANGES, AND CARBOHYDRATE UTILIZATION IN GLUCOSE AND LACTOSE MEDIA

rapidly than the lactose. The pH of the glucose cultures during the first 3.5 days was lower than that of the lactose cultures, but after the fourth day the reverse was true.

Various lots of crude whey concentrates were found to inhibit both growth and penicillin production. Lactose crystallized from such syrups was as satisfactory as a highly purified lactose. This situation was apparently due to the salt content of the whey concentrates. Various concentrations of NaCl were added to a nutrient medium containing 8.8 per cent of corn steep liquor. A 10 per cent or greater concentration of NaCl inhibited both growth and penicillin production (table 9). A similar salt toxicity was encountered when

high concentrations of corn steep liquor were neutralized with alkali, and with certain acid-hydrolyzed proteins.

The addition of a small amount of glucose to the basic medium containing lactose as the main carbohydrate source increased the penicillin yield in some cases and, furthermore, decreased the time required to attain the maximum yield. The value of adding 3 to 5 g of glucose per liter of medium seems to depend upon the glucose content of the corn steep liquor, which varies from about 2 to 12 per cent, depending upon the extent of the lactic fermentation occurring during manufacture. The advantage of adding glucose to the culture medium is most evident when highly fermented corn steep liquors (i.e., those having a low glucose content) are used.

Nitrogen sources and corn steep liquor. Various nitrogen sources, such as NaNO_3 , KNO_3 , $\text{Ca}(\text{NO}_3)_2$, $\text{Mg}(\text{NO}_3)_2$, NH_4NO_3 , NH_4Cl , and urea, were employed with and without corn steep liquor. Two to six units of penicillin per ml were obtained by using NaNO_3 , KNO_3 , $\text{Ca}(\text{NO}_3)_2$, or $\text{Mg}(\text{NO}_3)_2$ in a medium without corn steep liquor. Under similar conditions, only traces of penicillin were pro-

TABLE 9
Effect of various concentrations of NaCl on growth and penicillin production

NaCl—g per 100 ml.	0	1	2	3	4	5
Bulk of growth at 2 days—score.	1.3	0.9	0.5	0.1	0	0
Mycelium weight at 6 days—g per culture.	1.12	.61	.56	.41	.34	.26
pH of medium at 6 days.	7.2	6.9	6.1	5.4	4.6	4.3
Penicillin at 6 days—units per ml. ...	96	72	23	11	0	0

Culture medium: Lactose monohydrate, 4.4 per cent; corn steep liquor, 8.8 g per 100 ml; standard salts; and initial pH, 4.1.

duced by using NH_4Cl , NH_4NO_3 , or urea as nitrogen sources. In culture media containing 8.6 per cent of corn steep liquor, in addition to these individual nitrogen sources, no significant differences in penicillin production were detected. A near-optimum concentration of the inorganic source of nitrogen was attained by the use of 3.0 g of NaNO_3 per liter in a medium containing 7.5 to 10 per cent of steep liquor.

The crude yeast extract used by Abraham *et al.* (1941) was employed in small quantities primarily for the purpose of accelerating the growth of *P. notatum*, which develops slowly on unsupplemented Czapek-Dox medium. Experiments were undertaken to determine whether a similar result might be achieved by using corn steep liquor, a cheap, commercially available material which has long been employed as a supplement to nutrient media for the production of yeast; for the bacterial production of butanol and acetone (Legg and Christensen, 1932), sorbose (Wells *et al.*, 1939), 2-ketogluconic acid, and 5-ketogluconic acid (Stubbs *et al.*, 1940); and for the production of gluconic acid by *Aspergillus niger* (Moyer *et al.*, 1940).

The first tests of corn steep liquor in penicillin production were made by

using only 2 to 5 ml of this material per liter of Czapek-Dox medium. At these levels, corn steep liquor caused increased mold growth and gave a maximum yield of 10 units of penicillin per ml. In succeeding experiments, in which the concentration of corn steep liquor was increased, yields of 60 to 80 units of penicillin per ml were obtained in 6 to 8 days from a medium containing 3 per cent of glucose and 7.5 to 10 per cent of steep liquor.

The effect on penicillin yield, pH change, and fungus growth of various concentrations of corn steep liquor in a medium containing lactose is shown in table 10. The optimum concentration of corn steep liquor was between 7.5 and 12.5 per

TABLE 10

Effect of various concentrations of corn steep liquor on penicillin production

CORN STEEP LIQUOR CONCENTRATION	CULTURE AGE—DAYS					
	3	4	5	6	7	8
	Penicillin yield—units per ml					
<i>g per 100 ml</i>						
5	31	57	89	99	106	125
7.5	50	85	128	188	178	168
10	71	112	180	168	134	106
12.5	63	106	192	150	100	40
15	45	85	82	68	31	9
	pH of filtrates					
5	6.4	6.9	7.3	7.4	7.6	7.7
7.5	6.2	6.8	7.2	7.3	7.65	7.9
10	6.4	7.0	7.4	7.8	8.2	8.35
12.5	6.5	7.1	7.6	8.1	8.3	8.5
15	6.3	7.1	7.7	8.2	8.5	8.6
	Mycelium weight—g per culture					
5	0.54	0.72	0.87	0.81	0.93	0.95
7.5	0.67	0.96	1.15	1.21	1.21	1.16
10	0.91	1.17	1.37	1.34	1.30	1.18
12.5	0.99	1.33	1.46	1.40	1.24	1.04
15	1.00	1.36	1.46	1.50	1.19	1.13

Culture medium: Lactose, 5.5 g per 100 ml; glucose, 0.275 g per 100 ml; standard salts; media adjusted with KOH to initial pH 4.6.

Organism: *P. notatum* 1249.B21.

cent. The highest penicillin yield (192 Oxford units per ml) was obtained in 5 days with 12.5 per cent of steep liquor. Inhibition of penicillin accumulation was noted when 15 per cent of corn steep liquor was employed, although there was no inhibition of growth. The decrease in penicillin concentration occurring at 7 to 8 days was associated with a rise in alkalinity above pH 8.0 and with a decrease in the mycelium weight, and is attributed to a high initial concentration of corn steep liquor. When only 50 to 75 g of steep liquor per liter was employed, the alkalinity did not reach pH 8.0, and there was no significant decrease in penicillin content during the 8-day incubation period.

Other nutrient salts. In a culture medium containing 3.75 to 12.5 per cent of corn steep liquor, the concentrations of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KH_2PO_4 were increased and decreased from the amounts in the original Czapek-Dox medium without marked effect on growth or on penicillin yield; therefore, it appeared that corn steep liquor contains sufficient of these essential elements in available form. Although it was not obligatory to add $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KH_2PO_4 to corn steep liquor medium, these salts were generally added at concentrations of 0.25 g and 0.50 g per liter, respectively, in order to insure adequate nutrition regardless of the quality or quantity of steep liquor employed.

Hydrogen ion concentration. Corn steep liquor usually has a pH of about 3.9 to 4.1, some variation being encountered depending on the extent of lactic acid fermentation occurring during manufacture. Because of the presence of amino acids and other protein fission products, the steep liquor is highly buffered; thus, 100 ml of one sample of steep liquor required 161 ml of 1 N NaOH to raise the pH from 4.0 to 8.5, which is approximately the pH change occurring during the 7- to 8-day growth period of the fungus on a 10 per cent corn steep liquor medium.

TABLE 11
Effect of initial pH on penicillin production

Initial pH of medium.	5.7	5.25	4.9	4.4	4.1	3.95	3.8	3.65	3.4	3.25
Penicillin yield, units per ml.	154	148	143	132	124	113	103	88	64	38
Mycelium weight, g culture	0.98	0.99	1.00	1.03	1.13	0.95	0.95	0.95	1.04	1.02
pH of filtrates	7.8	7.8	7.75	7.5	7.4	7.0	6.9	6.75	6.5	6.0

Culture medium: Lactose, 4.4 g per 100 ml; corn steep liquor, 7.5 g per 100 ml; and standard salts.

Organism: *P. notatum* 1249.B21.

All data reported for 6-day-old cultures.

The net result of all reactions taking place in the nutrient medium is, therefore, to render the medium alkaline.

Since there was poor penicillin accumulation in a medium more acid than pH 5.0, it seemed that there might be an advantage in changing the initial pH of the medium. The pH of a series of media was accordingly raised above 3.95 with NaOH, or lowered with lactic acid. The correlation between penicillin production and various initial pH values of the medium is shown in table 11. The amount of fungus growth was not significantly affected within the pH range, 3.25 to 5.7. Raising the initial pH increased penicillin production, the maximum yield being obtained from cultures started at pH 5.7. Decreased penicillin production was observed in cultures started at low pH levels.

In another experiment, various concentrations of corn steep liquor were employed and the initial pH was raised with NaOH. A medium containing 10 per cent corn steep liquor and inoculated at pH 5.5 resulted in poor growth, as well as in poor penicillin production. This inhibition was apparently due to an increase in the salt concentration above the tolerance level of this organism, as

indicated in table 9. The level to which the initial pH of the medium can be raised is, accordingly, dependent upon the concentration of the corn steep liquor employed.

Addition of nutrients during the course of the fermentation. It is shown by data presented above that the quantity of penicillin in the broth decreases rapidly as the pH of the medium rises above 8.0. This decrease in penicillin yield was closely associated with the exhaustion of the carbohydrate, and with the beginning of mycelium autolysis (figure 1). There appeared to be a limit to the quantity of carbohydrate that could be included in the original culture medium, since an

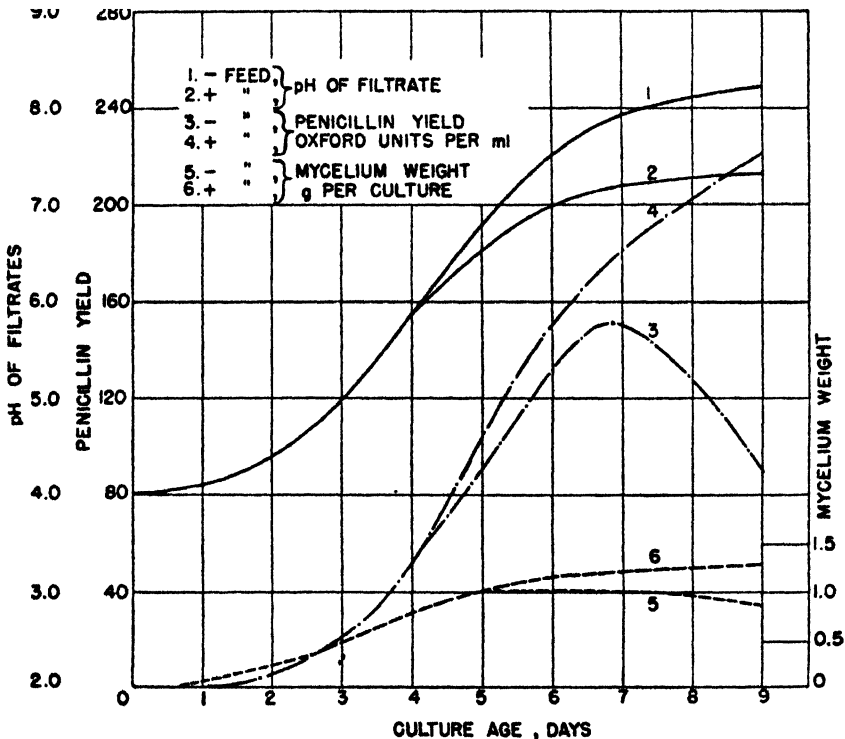


FIG. 2. EFFECT OF NUTRIENT ADDITION DURING FERMENTATION

FM-L-390-P-2

excess of this constituent caused a delay in penicillin accumulation. The increase in the pH of the medium was most rapid when the initial supply of available carbohydrate was low. Therefore, it seemed probable that the addition of carbohydrate during the course of the fermentation might inhibit the rise of the pH to a level at which penicillin was rapidly destroyed.

To study the effect of periodic addition of nutrients during the growth period, experiments were conducted using a near-optimum medium for penicillin production. This medium contained the standard salts, 10 per cent of corn steep liquor, and 4 per cent of lactose. Cultures were allowed to grow for 4 days. Then, beginning on the fourth day and daily thereafter, nutrients were

added by means of a sterile pipette. The control cultures received 1 ml of distilled water, and the fed cultures received 1 ml of a solution containing 0.20 g of lactose, 0.20 g of glucose, 0.010 g of corn starch, and 0.050 g of corn steep liquor per ml.

The effect of this feeding on penicillin yields, mycelial growth, and pH change are shown in figure 2. The addition of these nutrients caused an increase in penicillin as contrasted with the decrease in the control cultures. Penicillin yields of 220 units per ml were obtained on the ninth day, when the experiment was terminated. The pH of the fed cultures did not rise so rapidly as that of the control cultures, and there was an appreciable increase in fungus growth as a result of the added nutrients. The increase in penicillin yield was probably due both to a favorable pH and to a continued growth of the fungus.

Further experiments on the addition of nutrients showed that feeding 0.3 g of glucose per culture per day was nearly as effective as the foregoing combination of glucose, lactose, starch, and steep liquor. The addition of corn steep liquor alone was of little value in increasing penicillin yields. The addition of citric acid during the fermentation gave some pH control and an increase in penicillin production. The effect of pH control in all these cases is not clearly distinguishable from the effect of increased fungus growth. Addition of hydrochloric acid gave some pH control and prevented the decrease in penicillin yield normally occurring on the sixth or seventh day, but did not bring about appreciable increase in penicillin yield.

The optimum kind and amount of feeding, and the best time to begin it, have not been definitely established. These factors, it is believed, will vary considerably with the initial concentration of nutrients, the size of the culture flask, and the depth of the medium. Culture feeding may have some practical value in large pan cultures when mechanical stirring of the liquid is applied.

The Role of Corn Steep Liquor

The increase in penicillin yield due to the addition of steep liquor to the culture medium was so marked that the determination of the specific role of corn steep liquor became a very intriguing problem. It appeared that the steep liquor might supply any of a number of substances, such as trace elements, growth factors, amino acids, and possibly even building blocks for the penicillin molecule. Accordingly, experiments were planned to determine the effects of such substances when they were added to a medium containing no steep liquor.

Trace elements. *P. notatum* showed a typical, trace element requirement in a synthetic medium purified by the procedure as described by Steinburg (1935). A large number of trace elements, including zinc, iron, copper, molybdenum, manganese, and columbium were tested singly and in combination in the standard synthetic medium containing glucose but no corn steep liquor. None of these elements gave any response except zinc, which caused slight increase in growth and in penicillin production. Zinc has been included in the optimum medium containing corn steep liquor and lactose, but equally good results have been obtained when it was omitted.

P. notatum or *P. chrysogenum* gives good yields of gluconic acid when grown on the standard salt medium containing 0.5 per cent corn steep liquor and 5 to 25 per cent glucose. In such a medium, with or without CaCO_3 , the yield of gluconic acid is greatly reduced by the addition of zinc. The accumulation of gluconic acid could not be detected in a medium containing 4 to 5 per cent glucose and 4 to 12 per cent corn steep liquor, and, under such conditions, the addition of zinc exerted little if any effect on fungus growth or penicillin accumulation.

To test further the possibility of trace elements being responsible for the action of corn steep liquor, a sample of steep liquor was ashed, and the ash, redissolved in dilute HCl , was added to the standard synthetic medium referred to above. Only a slight increase in growth and penicillin yield was observed. It was concluded that this fungus requires some trace elements for growth but that the trace elements alone are not responsible for the effect of corn steep liquor on penicillin yield.

Growth factors. To test the effect on penicillin production of certain growth factors, pimelic acid, nicotinic acid, thiamine, inositol, pyridoxine, sodium pantothenate, biotin, and *p*-aminobenzoic acid were added separately and as a composite to the standard synthetic medium. These compounds caused no increase in growth or in penicillin formation, nor did they, when added to agar plate cultures, give any improvement in growth or sporulation. In contrast, a few drops of dilute corn steep liquor, so added, greatly stimulated growth and spore production. Accordingly it appeared that the steep liquor did not function primarily as a source of these growth factors.

Amino acids. The fact that growth and penicillin yield improved with the use of increased concentration of corn steep liquor as the sole source of nitrogen suggested the possible importance of its nitrogenous constituents. Since steep liquor contains many amino acids, the effect of a number of these, including glycine, alanine, tyrosine, methionine, tryptophane, valine, proline, histidine, phenylalanine, and β -alanine, was determined by employing the standard salt medium containing 3 per cent of glucose but no steep liquor. These amino acids, when used in 0.05 M concentrations, singly or in combinations, had little effect on growth and no effect on penicillin formation.

Fractionation of corn steep liquor. Diluted corn steep liquor was dialyzed through a collodion membrane. The dialyzable fraction, after concentration, was found to be as good as the untreated corn steep liquor for growth and penicillin production.

A prolonged extraction of corn steep liquor at pH 5.0 with *n*-butanol or ethyl ether failed to remove anything which, when added to the standard medium in place of steep liquor, materially favored penicillin production.

The addition of comparatively large volumes of acetone or ethyl alcohol to steep liquor yielded copious precipitates. In general, the greater the precipitation, the less potent the filtrate became for penicillin production. It was not possible, however, to obtain any clear-cut fractionation of an active principle.

Corn steep liquor was treated with sodium hydroxide to pH 9.0, the precipitate (probably consisting largely of phytin and basic phosphates) was filtered off, and the filtrate acidified to pH 4.3 with hydrochloric acid. Steep

liquor thus treated was as good as, but not better than, the untreated liquor. (In this case, the effect of high salt concentration was not apparent because of the use of only 4 per cent of steep liquor.) When the untreated steep liquor medium (containing 7.5 per cent steep liquor) was partially neutralized to pH 5.6 and steam-sterilized, a heavy precipitate formed and remained in the flasks during the fermentation. Such cultures gave better penicillin yields than those started at pH 4.0, in which there was much less precipitation. Removal of the precipitate from such cultures before inoculation did not cause a decrease in penicillin yield.

Substitutes for corn steep liquor. Since the work on corn steep liquor suggested the importance of amino acids and protein split products in penicillin production, other crude nitrogen sources were investigated. Finely ground soybean meal, fish meal, or a whole corn mash gave only slight increase in fungus growth and penicillin production. Various acid-hydrolyzed protein preparations from corn, wheat, and soybeans were all inferior to corn steep liquor when used on the basis of equal total nitrogen. It is believed that, to some extent, the ineffectiveness of these acid-hydrolyzed preparations was associated with their high salt content.

Trypsin-hydrolyzed casein, however, gave promising results when used in 1, 2, and 3 per cent concentrations (based on the dry casein), yielding in six days, 22, 20, and 19 units of penicillin per ml, respectively, as compared with 80 units per ml with 7.5 per cent corn steep liquor and 4 per cent glucose. However, concentrations greater than 3 per cent had a toxic effect and did not lead to increased penicillin yields. Subsequent to these tests, it was ascertained that British investigators had used acid-hydrolyzed casein, obtaining penicillin yields similar to those given above. Milk, whole or skimmed, has some value for penicillin formation, but probably not enough to merit commercial exploitation. By using 80 per cent of skimmed milk as the only nutrient in the medium, penicillin yields of 13, 20, 19, 26, and 31 units per ml were obtained at 4, 5, 6, 7, and 8 days, respectively.

Bacto-peptone, some commercial grades of peptone, and Difco dried yeast compound were ineffective in promoting penicillin production, although all of these products supported a good growth of the organism. The pH rise in the media was much slower than that encountered with corn steep liquor.

A malt syrup, employed in concentrations ranging up to 10 per cent by weight, produced maximum penicillin yields of 30 units per ml, which was approximately one third of the yield obtained with a corresponding weight of corn steep liquor. Very heavy precipitates were encountered upon steam sterilization. Portions of this malt syrup, when sterilized by filtration, inhibited all penicillin production without any inhibition of growth.

A barley and a soybean steep liquor, when tested over a wide range of concentration, proved worthless for penicillin production.

DISCUSSION OF RESULTS

A culture medium for good penicillin production must not only provide nutrients suitable for rapid fungus growth and penicillin formation, but must also provide conditions under which the penicillin is not too rapidly destroyed. The

amount of penicillin which accumulates in the medium is, obviously, the difference between the amount produced and the amount destroyed. When low penicillin yields are obtained on a given medium, it should not necessarily be assumed that only a small amount of penicillin is formed by the fungus. In many cases it is probable that only a small amount of penicillin survives some unfavorable condition prevailing in the culture. Important factors influencing the stability of penicillin during fermentation are the temperature and pH of the medium. The instability of penicillin to numerous chemical and physical factors was recognized by early investigators, particularly in connection with isolation and purification procedures. Recent investigations by Benedict *et al.* (1945) on highly purified penicillin preparations have shown that penicillin inactivation is a function of temperature and pH, and that the greatest stability in solution is exhibited at pH 6.0. It is to be noted that the temperature and pH conditions which necessarily prevail in the culture medium throughout most of the growth period are not optimum for penicillin stability.

The data obtained in the course of these nutritional investigations indicate that penicillin is produced during the active growth of the fungus and is not an autolytic product released after growth has ceased. If this is correct, the the greatest quantity of penicillin should accumulate when the maximum growth occurs at or near pH 6.0, where penicillin is most stable. As shown in figure 1, penicillin formation can be detected by the time the pH has risen to 4.5. At this pH and temperature the half-life of pure penicillin is about 60 hours. In the culture liquors it is materially less stable. It is therefore probable that outside the pH limits of 5.0 to 7.5 there is substantial loss of penicillin. Near-optimum growth-pH- penicillin relationships are believed to exist when the greater part of the fungus growth occurs between pH 5.0 and 7.5. In the lactose medium (figure 1), 72 per cent of the growth occurred between pH 5.0 and 7.0, and in the glucose cultures only 31 per cent of the growth occurred in this range, 69 per cent of it occurring below pH 5.0.

There are several factors involved in the pH rise during the course of the fermentation. A typical corn steep liquor has a high buffer capacity (100 g may require as much as 160 ml of 1 N alkali to raise the pH from 4.0 to 8.5). During the fermentation the pH normally rises from 4.0 to over 8.0. This change is believed to be due both to assimilation of the lactate in the corn steep liquor (leaving the cation), and to the formation of alkaline products of metabolism such as ammonia. Both processes probably occur simultaneously.

It has been demonstrated that lactic acid or Ca-lactate can serve as a carbon source for this fungus in a medium containing corn steep liquor. Thus it would appear logical to assume that the lactate occurring in the corn steep liquor would be as readily assimilated as lactate which had been added to the culture medium. A pH rise would result from the utilization of the lactate and from the release of alkaline or alkaline earth elements originally combined as salts of lactic acid. The accumulation of sodium ions from the utilization of the nitrate ion of NaNO_3 will also account for part of the pH rise.

The rapidity and extent of the pH rise is associated with the amount of corn steep liquor and the amount and kind of carbon source supplied. Other inves-

tigators of fungal or bacterial metabolism have shown that deamination of protein degradation products may occur, especially when the available supply of readily assimilable carbohydrate is low, or after it has been exhausted. Kendall and his associates (1913) in their studies on bacterial metabolism, concluded that utilizable carbohydrates protect proteins from bacterial breakdown. Waksman (1917), in his investigations of the influence of available carbohydrate upon ammonia accumulation, demonstrated "the protein sparing action of the carbohydrates" in pure cultures of *Aspergillus niger* and *Citromyces glaber*. He found that ammonia accumulation was greatest when the supply of carbohydrate was low or after it had been exhausted. He concludes that, when available carbohydrates are present, the fungus utilizes all the nitrogen split off from the protein for its own metabolism; in the absence of carbohydrate, the protein molecule is attacked not only for its nitrogen but also for its carbon content. Butkewitsch (1922) showed with cultures of *Citromyces* that 75 per cent of the total nitrogen accumulates as ammonia in the culture medium when peptone is used as the sole nitrogen and carbon source. It has been shown in this paper that the pH rises more rapidly in a medium in which corn steep liquor is the sole source of carbon than in one in which glucose is used to supplement the corn steep liquor. It has also been shown that the pH rise in a steep liquor medium is less rapid when the supply of carbohydrate is increased. The addition of glucose during the fermentation also inhibits the rapidity of the pH rise. These results suggest that the accumulation of ammonia, through deamination of amino acids, can play an important part in the pH rise during the fermentation.

A difference in pH rise occurs with the various carbon sources. There is a more rapid rise in pH in the lactose cultures during the first part of the fermentation, and a slower rise later, than there is in the glucose cultures. These differences in pH change are believed to be due to differences in the availability of the carbon sources. It has been shown that lactose does not support growth of the fungus in the standard salt medium. Lactose in the presence of corn steep liquor is more slowly utilized than is glucose. A partial carbohydrate starvation may occur during the first few days of growth, before the lactose is rapidly hydrolyzed. During this period, ammonia from deamination apparently accumulates faster than it can be assimilated, resulting in a rise in pH. Starch also must undergo enzyme hydrolysis, and, in a manner similar to that of lactose, its use results in a slow rise in pH during the first few days of culture incubation.

P. notatum produces gluconic acid from low concentrations of glucose in a medium low in nitrogen, but in the standard medium with 3 to 4 per cent glucose and 6 to 8 per cent corn steep liquor, sufficient gluconic acid has not been detected to account for the slow rise in pH. So far as has been observed, *P. notatum* does not produce appreciable amounts of any organic acid from lactose under any nutrient conditions.

The use of lactose or starch as a carbon source provides a more satisfactory range of pH change during the fermentation period than can be obtained with the more readily available carbon sources such as glucose, sucrose, glycerol, or sorbitol. With lactose or starch, a higher percentage of fungus growth occurs

within a pH range favorable to the accumulation of penicillin than occurs with the more readily available carbon sources. Such slowly available carbon sources as lactose and starch appear actually to prolong the productive life of the culture.

The role of corn steep liquor in the production of penicillin appears to be complex and is not clearly understood. It obviously supplies nutrients, chiefly nitrogenous compounds which favor a rapid growth of the fungus. Nitrogenous compounds such as peptone, soybean meal, fish meal, Difco yeast compound, and a whole corn mash also support a fair fungus growth but give lower penicillin yields than were obtained with corn steep liquor. Investigations of such compounds have not been complete enough to establish a critical composition difference from corn steep liquor. The role of the corn steep liquor in maintaining a proper pH change appears to be just as important as the maintenance of a vigorous fungus growth.

The value of these nitrogenous compounds may depend upon the extent to which the protein molecule is broken down. This occurs through the following stages: protein, peptones, polypeptides, and amino acids. The amino acids may be broken down by deamination or decarboxylation. Berger *et al.* (1937) made a comparison of the proteolytic activities of some common molds. The species of *Penicillium* showed low proteinase and dipeptidase activity, but they had a fairly high aminopolypeptidase activity. Kirch (1939) found that *P. luteum-purpureogenum*, when grown on soybean meal for 30 days at 23 to 26 C, showed weak proteolytic activity, and the presence of amino acids could not be detected. If *P. notatum* is correspondingly weak in its proteolytic activity, the value of an organic nitrogen supplement may be related to its content of amino acids, which this fungus can utilize.

A cheaper and more satisfactory organic nitrogen supplement than corn steep liquor has not been found for penicillin production. The use of corn steep liquor, with lactose or starch as component parts of the nutrient medium, has resulted in greatly increased penicillin yields and the establishment of commercial production.

SUMMARY AND CONCLUSIONS

Several culture methods can be utilized to produce spores for the inoculation of production cultures. Spores for laboratory inoculations can be readily grown on agar slants or on petri dish cultures. The use of dry spores mixed with a floating and spreading agent, such as whole-wheat flour, has given very satisfactory results in uniformity of surface growth and penicillin yield.

In the search for a better organism for penicillin production in surface culture, none was found superior to one of the descendants of the original Fleming strain which had been freed, insofar as possible, from degenerate, mutant strains. Continual precautions must be taken to guard against the appearance of these inferior forms. This special strain (NRRL 1249.B21) is now widely used for the industrial production of penicillin by the surface culture method.

The yield of penicillin produced in surface cultures by *Penicillium notatum* Westling, has been increased from 2 to 6 to over 200 units per ml. The addition of corn steep liquor to the culture medium greatly increases the penicillin yield.

The use of lactose or starch is found to give higher penicillin yields than can be obtained with glucose, sucrose, sorbitol, or glycerol. The use of strain NRRL 1249.B21 in a culture medium containing corn steep liquor and lactose has given penicillin yields of 190 units per ml in 5 days. The addition of nutrients during the course of the fermentation has further increased the yield up to 220 units per ml.

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PENICILLIN

IX. THE LABORATORY SCALE PRODUCTION OF PENICILLIN IN SUBMERGED CULTURES BY *PENICILLIUM NOTATUM* WESTLING (NRRL 832)¹

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Penicillin was first produced by cultivation of the fungus *Penicillium notatum* Westling on the surface of a liquid nutrient, such as Czapek-Dox glucose medium. Such a method of cultivation is very laborious, costly, and time-consuming when practiced on a large scale. Huge numbers of bottles or pans must be washed and sterilized, relatively small volumes of nutrient media must be dispensed into individual containers, and each container with its allotment of medium must be sterilized and inoculated. The incubation period for such cultures is usually 6 to 12 days, and, at the conclusion of the fermentation, considerable hand labor is required to remove the penicillin-containing liquors from the numerous fermentation vessels and from the fungus mycelium.

It was obvious that a more economical fermentation process would result from growing the mold submerged and uniformly distributed in vats or tanks such as are used in other fermentation industries. Submerged mold fermentation processes have been previously used for the production of gallic acid (Calmette, 1902), gluconic acid (Moyer *et al.*, 1940), and lactic acid (Ward *et al.*, 1938), and the adaptation of this method to the cultivation of *P. notatum* appeared to offer a means of decreasing the labor involved, of decreasing the fermentation time, and of increasing the penicillin yield.

This paper deals with the selection of a strain of *P. notatum* Westling suitable for the production of penicillin in submerged culture, and with investigations of nutrient media and culture conditions as nearly optimal as possible for this procedure.³ (*P. notatum* NRRL 832 was originally selected and evaluated for submerged penicillin production by the senior author.)

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³The results of this work have been communicated monthly and bimonthly since March, 1942, in a series of restricted reports to Dr. A. N. Richards, chairman, Committee on Medical Research, Office of Scientific Research and Development, who, in turn, sent copies to all penicillin producers and to many research groups in this country and abroad. Owing to the strategic significance of penicillin, publication of papers covering this research has been delayed.

METHODS AND MATERIALS

The fungus was grown in the submerged condition in 300-ml Erlenmeyer flasks which were shaken continuously on Ross-Kershaw shaking machines. In this machine the flasks are secured to a flat table which is mounted eccentrically and revolved at 200 cycles per minute. This movement imparts a swirling motion to the contents of the flasks and serves both to agitate and aerate the medium. Since the flasks are plugged with cotton, gas diffusion into and out of the flasks occurs readily. All cultures were maintained at 24 C. Samples were withdrawn for penicillin assay and pH determinations by means of a sterile, wide-mouthed pipette.

Assays were made by the cylinder-plate method originated by Abraham *et al.* (1941), and modified by Schmidt and Moyer (1944). Determinations of pH were made electrometrically.

The nutrient salts, unless otherwise specified, were $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; KH_2PO_4 , 0.50 g; NaNO_3 , 3.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.044 g; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.020 g per liter of medium. This concentration of salts will be referred to as the standard nutrient salts. The CaCO_3 was always sterilized dry in separate containers and added just prior to inoculation. The corn steep liquor employed contained 50 to 55 per cent total solids, 5 to 6 per cent free sugar calculated as glucose, and an acidity corresponding to pH 4.0.

Two types of inoculum were employed, namely, ungerminated spores used dry or wetted in a 0.1 per cent soap solution, and germinated spores in the form of clumps or tiny pellets. The pellet inoculum was prepared by developing mycelium from spores, in submerged culture, in a medium containing, in addition to the standard nutrient salts, 30.0 g of lactose and 55 ml of corn steep liquor per liter. One gram of sterile CaCO_3 and 125 ml of medium were employed for each 300-ml flask. In seeding flasks with spores, a suspension of spores from an agar slant (in a 6- by 1-inch test tube) was made in 60 ml of a 0.1 per cent soap solution; 10 ml of this suspension were used to inoculate each culture for pellet production. When it was desired to start the fermentation with ungerminated spores, 1 ml of such a spore suspension were used for direct inoculation of production cultures. After 2 to 3 days on the Ross-Kershaw shaker, the pellets were 0.5 to 1.0 mm in diameter, and the solution had a penicillin content of 8 to 10 units per ml and a pH of 7.6 to 7.8. Usually 5.0 to 7.5 ml of this pellet preparation were employed to inoculate a production culture.

EXPERIMENTAL RESULTS

Selection of the organism and sporulation media. The first experiments on penicillin production in submerged culture were made with the following strains: the Fleming strain of *P. notatum* supplied by Dr. Florey of Oxford University, and a strain also derived from the Fleming strain but obtained from the Squibb Institute for Medical Research, the latter now being designated as *P. notatum* NRRL 1249 in our culture collection. Various combinations of nutrients, includ-

ing carbon and nitrogen sources, trace elements, CaCO_3 , and corn steep liquor, were investigated. The highest penicillin yield obtained was only 14 Oxford units per ml.

A limited survey of other strains of *Penicillium*, which had given promising penicillin yields in surface cultures, was made in submerged culture by using a medium containing lactose and corn steep liquor. It was found that, under these conditions, *P. notatum* NRRL 832 was superior to the other strains for penicillin production. The origin of this strain has been reported by Raper, Alexander, and Coghill (1945).

After improvements had been made in the culture conditions, other tests were made, in which were used various strains of *P. notatum*, *P. chrysogenum*, and allied species which were available. The results of a part of this survey are

TABLE 1

Comparison of penicillin yields obtained with strains of P. notatum and P. chrysogenum in submerged cultures

ORGANISMS*	CULTURE AGE—DAYS				
	3	4	5	6	7
	Units per ml				
NRRL 832	14	27	34	54	53
NRRL 838	6	10	15	16	21
NRRL 824	14	18	28	30	27
NRRL 807	4	8	12	15	17
NRRL 811	4	7	7	6	6

Culture medium: Lactose 20 g; standard salts; corn steep, 40 ml; and distilled water to make 1 L. CaCO_3 , 1.5 g per culture. All inoculated with ungerminated spores.

* 832, *P. notatum*, obtained from the Thom collection as no. B-69 (not related to Fleming strain).

838, *P. cyaneo-fulvum*, obtained from the Thom collection as no. 5221.

824, *P. notatum*, obtained from the Thom collection as no. 5112.1 from the Fleming strain received by Thom in 1930.

807, *P. chrysogenum*, obtained from the Thom collection as no 26.

811, *P. chrysogenum*, obtained from the Thom collection as no 5034.11.

shown in table 1. Under the conditions indicated, *P. notatum* NRRL 832 again gave the highest penicillin yields, and strain NRRL 824 showed some promise. Because of the apparent superiority of *P. notatum* NRRL 832, it was used in all subsequent studies on submerged penicillin production.

P. notatum NRRL 832 rapidly developed a heavy crop of spores on a fairly wide range of nutrient media. One of the best agar media was the one recommended for *P. notatum* NRRL 1249.B21 by Moyer and Coghill (1945). For the large-scale production of spores, wheat bran moistened with a 2 per cent solution of corn steep liquor was also found satisfactory, as were small cubes of fresh whole-wheat bread. A good crop of spores developed in 4 to 5 days at 24 to 26 C on either the bread or bran medium.

Carbohydrate sources. One of the best media for submerged cultures has the following composition:

Corn steep liquor ⁴	40.0	ml
Lactose monohydrate.....	27.5	g
NaNO ₃	3.0	g
MgSO ₄ ·7H ₂ O.....	0.25	g
KH ₂ PO ₄	0.50	g
ZnSO ₄ ·7H ₂ O.....	0.044	g
MnSO ₄ ·4H ₂ O.....	0.020	g
Glucose monohydrate.....	3.0	g
Distilled water to make 1 liter		

Previous studies (Moyer and Coghill, 1945) of the production of penicillin in surface culture showed that sorbitol, glucose, and sucrose were inferior to lactose as carbon sources, despite the fact that the fungus grows readily on media containing any of these compounds. When *P. notatum* NRRL 832 was cultivated (submerged) in media similar to that described above, except that lactose was replaced by similar concentrations of glucose, sorbitol, or sucrose, and that 1.5 g of CaCO₃ was added to each flask, only about half as much penicillin was produced as when the lactose medium was employed. At the age of 3 days all of the cultures which had been inoculated with ungerminated spores contained a large number of pellets 1 to 2 mm in diameter. During the first 3 days the pH rose more slowly in the glucose, sucrose, and sorbitol than in the lactose cultures, but thereafter it continued to rise more rapidly in the lactose cultures. After the pH had risen above 8.0, the penicillin content of the broth decreased sharply.

The effect of various concentrations of lactose on penicillin yield is shown in table 2. The optimum lactose concentration under these conditions appeared to be between 2 and 3 per cent. Cultures having lower lactose concentrations showed a rapid rise in the pH of the broth; a more gradual pH rise was observed in cultures containing 3 and 4 per cent lactose. It is believed that the slower rise in pH is more favorable for penicillin production.

Corn steep liquor. Preliminary experiments conducted in media containing varying quantities of concentrated corn steep liquor indicated that at least 40 ml of steep liquor per liter is required to obtain high yields of penicillin. Data obtained from an experiment designed to compare the effects of using 40 and 60 ml of steep liquor per liter of medium and two different lactose concentrations are presented in table 3. The higher yields of penicillin were obtained from media containing the smaller quantity of steep liquor, regardless of lactose concentration. There was heavier growth in the cultures containing the most steep liquor; however, the pellets were not so compact, but more nearly filamentous, so that the entire liquid was very viscous. The pellets produced in the medium containing 40 ml of steep liquor were much more compact.

⁴The commercial product, 30° Baume, containing 50 to 55 per cent total solids was used, unless otherwise noted.

No significant difference in pH change was observed in the cultures containing 20 or 25 g of lactose per liter. The cultures containing 40 ml of corn steep liquor

TABLE 2
Effect of different concentrations of lactose on penicillin yields

LACTOSE	MEASUREMENTS MADE ON SUCCESSIVE DAYS				
	3	4	5	6	7
	Units per ml				
%					
1	23	36	34	27	19
2	26	60	74	61	47
3	26	60	72	80	80
4	26	60	70	73	82
	pH of media				
1	7.8	7.9	8.1	8.4	8.5
2	7.6	7.5	7.8	8.0	8.1
3	7.6	7.5	7.6	7.8	7.8
4	7.5	7.5	7.6	7.8	7.8

Culture medium: Corn steep, 40 ml; and standard salts, with 1.5 g CaCO₃ per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

TABLE 3
Effect of concentration of corn steep liquor on penicillin yields

CORN STEEP	LACTOSE	MEASUREMENTS MADE ON SUCCESSIVE DAYS					
		3	4	5	6	7	8
		Units per ml					
%	g per L						
4	20	12	20	40	73	80	68
6	20	11	18	27	34	47	
4	25	32	47	59	80	80	
6	25	22	34	37	45	59	
		pH of media					
4	20	7.5	7.8	7.6	7.5	7.8	8.0
6	20	7.5	7.8	7.8	7.85	7.8	
4	25	7.5	7.7	7.5	7.7	7.8	
6	25	7.6	7.7	7.8	8.0	8.0	

Culture medium: Standard salts, with 1.5 g CaCO₃ per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

per liter showed a decrease in pH on the fifth and sixth days, but such a decrease was not observed in the cultures containing 60 ml of corn steep liquor. It is believed that the poorer yields of penicillin which resulted when higher concentrations of corn steep liquor were employed were due to an increased supply of

readily available carbon. The heavy viscous growth on such cultures, as contrasted with the cultures having less growth, appears to be correlated with insufficient aeration.

The effect of CaCO_3 . The effect of CaCO_3 on fermentations conducted in media containing varying quantities of corn steep liquor and inoculated with ungerminated spores is shown in table 4. It was found in all cases that cultures containing CaCO_3 produced more penicillin than those without it, and that the pH rise during the fermentation was much less than in the cultures lacking it. That better penicillin yields resulted when 6 per cent of steep liquor was used than when a 4 per cent concentration was employed is probably due, at least in part,

TABLE 4

Effect of different concentrations of corn steep liquor with and without CaCO_3 on penicillin yields

CORN STEEP LIQUOR	CaCO_3 PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS		
		4	5	6
		Units per ml		
%	g			
2	0	6	8	15
2	1.0	9	15	20
4	0	8	12	17
4	1.0	17	27	47
6	0	5	11	17
6	1.0	23	36	60
		pH of media		
2	0	6.3	6.5	7.0
2	1.0	7.3	7.2	7.4
4	0	6.2	6.4	6.9
4	1.0	7.2	7.3	7.4
6	0	6.5	6.2	6.4
6	1.0	7.5	7.4	7.6

Culture medium: Standard salts, with 25 g lactose per L. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

to the fact that this particular lot of corn steep liquor contained only 46 per cent total solids instead of the 54 per cent usually present.

The principal function of CaCO_3 is probably the partial neutralization of the lactic acid present in the corn steep liquor, resulting in the adjustment of the pH of the medium to a more favorable level. The unneutralized lactose corn steep liquor medium has a pH of about 4.0; this pH is increased to 5.3 and 5.4 upon addition of CaCO_3 . There appears to be some degree of specificity among neutralizing agents, since media adjusted to pH 5.0 to 7.0 with KOH have not resulted in such good yields as have been obtained on media treated with CaCO_3 .

A precipitate is always formed upon heat sterilization of corn steep liquor media, the quantity of this precipitate being much increased in media to which CaCO_3 is added. In media containing the heavy precipitates and CaCO_3 ,

formation of the pellet type of growth is more pronounced, and within 3 days the pellets contain all of the undissolved CaCO_3 within their hyphal meshes and most of the precipitated fraction of the corn steep liquor.

As shown in table 5, the addition of CaCO_3 to surface cultures of *P. notatum* NRRL 832 resulted in decreased mycelial growth and decreased penicillin yields. The penicillin yields are much lower than those obtained on the same medium in the shaken flasks. The precipitate in these surface cultures remains on the bottom of the culture vessels and is never in contact with the surface mycelium, whereas in the submerged cultures the mycelial pellets are formed around the

TABLE 5
*Effect of CaCO_3 on penicillin yields by *P. notatum* NRRL 832 in surface cultures*

LACTOSE	CaCO ₃ PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS				
		3	4	5	6	7
Units per ml						
%	g					
2	0	7	15	20	23	23
2	1.0	5	8	8	9	10
4	0	7	9	23	27	24
4	1.0	5	7	8	7	7
pH of media						
2	0	5.3	5.9	6.8	7.3	7.8
2	1.0	6.9	7.0	7.1	7.2	7.5
4	0	5.0	5.5	6.8	7.1	7.3
4	1.0	6.9	6.9	6.7	6.6	6.7
Dry weight fungus per culture						
2	0	.42	.50	.57	.52	.53
2	1.0	.25	.33	.42	.41	.50
4	0	.45	.59	.75	.77	.79
4	1.0	.24	.34	.44	.44	.45

Culture medium: Standard salts; corn steep, 40 ml per L. Inoculated with ungerminated spores. Culture size: 50 ml in 200-ml Erl. flasks.

precipitated particles. It is not unlikely that the CaCO_3 removes from the medium some essential factors or trace elements which are needed for good fungus growth and penicillin formation. In surface cultures containing 8 per cent of corn steep liquor and no CaCO_3 , much higher penicillin yields have been obtained with this fungus (Moyer and Coghill, 1945).

Other components of the nutrient medium. The concentrations of magnesium sulfate and mono-potassium phosphate have been increased up to four times the amounts specified in the standard submerged nutrient medium without a corresponding increase in penicillin yields. These salts have been omitted in a medium containing 4 per cent by volume of corn steep liquor, with no resultant decrease in penicillin yields. These results indicate that inorganic ions equiv-

alent to these nutrient salts are present in sufficient amounts in the corn steep liquor; therefore the addition of these salts to a corn steep liquor medium is not essential.

Better penicillin yields have been obtained with NaNO_3 than with urea or any of the ammonium salts. The concentration of NaNO_3 has been decreased to 1.5 g per liter without a decrease in penicillin yields. However, lower yields resulted when NaNO_3 was omitted or increased much beyond 3.0 g per liter.

Media containing corn steep liquor, crude lactose, and tap water do not show any superiority after the addition of zinc or maganese ions; hence, the addition of these ions is regarded as optional.

The addition of small amounts of glucose to the culture medium did not result in decrease in penicillin yields; on the contrary, when highly fermented corn steep liquor (which was low in glucose) was employed, significant increases in penicillin yields were obtained by the addition of 3 to 5 g of glucose monohydrate per liter of medium. *P. notatum* will not grow readily in a synthetic medium in which lactose is the sole carbon source. The addition of 2 to 3 ml of corn steep liquor per liter to a synthetic medium will enable the fungus spores to germinate and to attack the lactose. Under such conditions the addition of a small amount of glucose (0.3 to 0.5 per cent) along with the steep liquor markedly increases the growth rate. Thus, the beneficial action of glucose in submerged cultures may be related to the rapid establishment of a vigorous vegetative growth which can elaborate the lactose-attacking enzyme. If the steep liquor employed is not the highly fermented type and if it contains the normal quantity of sugar, the addition of glucose is unnecessary. In a corn steep liquor lactose medium, the readily available carbohydrates are believed to be practically exhausted before the lactose is utilized. A partial carbohydrate starvation may exist before the lactose is utilized.

Addition of nutrients during fermentation. An increase in penicillin yield in surface cultures of *P. notatum* NRRL 1249.B21 can be effected by the addition of nutrients during the fermentation, as demonstrated by Moyer and Coghill (1945). The periodic, or continuous, addition of nutrients to the submerged cultures is easier and more practical than such addition to surface cultures. It is obvious that innumerable combinations of nutrients and feeding schedules are possible, but in the limited time available it has not been practicable to make an exhaustive study of all these factors. However, it was found that the addition of certain nutrient components, principally glucose, was advantageous. The addition of corn steep liquor alone was not sufficient to prevent a rapid rise in the pH of the medium, with its concomitant decrease in penicillin potency, when the alkalinity of the medium became greater than pH 8.0. The effect of adding steep liquor alone, or glucose plus steep liquor, during the course of the fermentation is shown in table 6. Beginning on the fourth day, 5-ml samples were withdrawn daily for assay and pH determination, and 5-ml portions of the specified feed solutions were added. At 4 days, this series of cultures showed a slightly better than average penicillin yield. All cultures showed excellent pellet formation, and there was appreciable foam in the flasks. The formation of yellow pigment

was not intense. On the seventh and eighth days, the cultures receiving the glucose steep liquor feed showed appreciably more growth and slightly larger pellets than did the control cultures. The cultures receiving corn steep liquor alone differed only slightly from the controls, which received distilled water. The penicillin yields in the control cultures and in the cultures receiving the corn steep liquor feed reached a maximum at 6 to 7 days, and dropped steadily thereafter as the pH rose above 8.0. In contrast, those cultures receiving glucose plus steep liquor showed a steady increase in penicillin content until a maximum yield of 127 Oxford units per ml was reached on the ninth day. The pH of this group of cultures did not rise above 8.0 until the eleventh day.

TABLE 6
Effect of adding nutrients during the fermentation on penicillin yields

	MEASUREMENTS MADE ON SUCCESSIVE DAYS						
	4	5	6	7	8	9	11
	Units per ml						
Feed A.	51	48	52	59	38	30	11
Feed B.	50	70	74	68	42	36	18
Feed C.	50	68	78	91	120	127	102
	pH of media						
	4	5	6	7	8	9	11
	pH of media						
Feed A.	7.5	7.9	8.15	8.25	8.4	8.6	8.7
Feed B.	7.6	7.7	8.15	8.2	8.2	8.45	8.6
Feed C.	7.6	7.65	7.8	7.9	7.9	7.9	8.1

Culture medium: Lactose, 20 g; corn steep liquor, 40 ml; standard salts; and distilled water to make 1 L; CaCO_3 , 1.5 g per culture. Inoculated with ungerminated spores of *P. notatum* NRRL 832.

First feed on fourth day, and daily thereafter.

Feed A: 5 ml of distilled water.

Feed B: 5 ml containing 0.2 ml of steep liquor.

Feed C: 5 ml containing 0.9 g glucose and 0.2 ml steep liquor.

The use of a pellet inoculum. When the inoculum used for submerged cultures consisted of ungerminated spores, a large proportion of the tiny colonies, resulting from germination of the spores, washed up on the walls of the flask just above the liquor level. This made it necessary to remove the flasks from the shaking machine several times during the first 3 days of incubation in order to wash the colonies back into the liquid by a vigorous shaking. When this was not done, a solid band of mycelium was formed on the flask walls, and the number of pellets in the broth was greatly reduced. After the pellets had attained a diameter of about 1 mm they were not so readily washed up on the sides of the flasks. In order to prevent this wall growth, it seemed advisable to make the inoculation with a preformed pellet inoculum. It was hoped that the use of this pellet inoculum would also result in greater uniformity within a culture series and would effect a decrease in the time required for the fermentation.

A pellet inoculum may be prepared by heavily seeding ungerminated spores directly into the standard lactose steep liquor production medium, followed by a 2- to 3-day period of shaking. It is advantageous to modify this medium slightly for more rapid growth and pellet formation, as shown in table 9. At 2 days the pH of either pellet inoculum was 7.8, and the broth assayed 8 to 10 Oxford units per ml.

Ten- and twenty-ml portions of a pellet inoculum, grown for 2 days in the standard lactose steep liquor production medium (1.5 g of CaCO_3 per 125 ml of medium), were used to seed cultures containing the standard medium found to be optimum when ungerminated spores were used as the inoculum. The tendency for the fungus growth to accumulate on the flask walls was greatly reduced, re-

TABLE 7

Effect of CaCO_3 on penicillin yields with a pellet inoculum in glucose and lactose media

CARBON SOURCE	CaCO_3 PER CULTURE	MEASUREMENTS MADE ON SUCCESSIVE DAYS			
		3	4	5	6
		Units per ml			
2% Lactose	0	17	24	61	87
	1.0	18	14	3	0
2% Glucose	0	8	17	26	19
	1.0	20	31	41	22
2% Lactose	0	7.6	7.65	7.7	8.0
	1.0	8.1	8.35	8.4	8.6
2% Glucose	0	6.3	7.6	8.1	8.5
	1.0	7.2	7.9	8.15	8.5

Culture medium: Corn steep liquor, 40 ml; $\frac{1}{4}$ strength of standard salts; and distilled water to make 1 L. Ten ml of a 3-day-old pellet inoculum used per production culture.

sulting in uniformity in the culture series with respect to pellet size and total amount of growth. The most conspicuous result was the rapid rise in the pH, which reached 8.1 to 8.3 in 4 days. The highest penicillin yield was 35 units in 5 days.

Since this low penicillin yield seemed to be associated with a too rapid increase in the alkalinity of the medium, another series of cultures was prepared, using 2 per cent glucose or lactose, both with and without CaCO_3 . The inoculum consisted of 10 ml of a pellet suspension, similar to that described above (table 7). Low penicillin yields were again obtained in the lactose cultures containing CaCO_3 , whereas much better yields were obtained in those cultures lacking it; the pH of the medium did not rise so rapidly in the former cultures. Better penicillin yields were obtained in the glucose cultures when CaCO_3 was added to the medium.

It has been observed that the increase in the size of the pellets in the production medium depends upon the quantity of inoculum employed. When it is low, very large pellets (4 to 6 mm in diameter) are formed. The best penicillin yields have been obtained when 5 to 7.5 ml of pellet inoculum has been used per production culture, the pellets attaining a diameter of 2.5 to 3.0 mm. The effect of using 5 and 15 ml of pellet inoculum is shown in table 8. In this series, no CaCO_3 was added, and half the standard salt concentration was used. The better

TABLE 8
Effect of different amounts of pellet inoculum on penicillin yields

PELLET INOCULUM PER CULTURE	MEASUREMENTS ON SUCCESSIVE DAYS					
	3	4	5	6	7	8
	Units per ml					
ml						
5	21	39	88	91	99	78
15	18	22	25	41	37	26
	pH of media					
5	7.7	7.5	7.6	7.6	7.7	8.0
15	8.0	7.8	7.7	7.7	7.9	8.2
	Average pellet diameter, mm					
5	3	3	3	3	3	3
15	2	2	2	2	2	2
	Formation of yellow pigment in medium*					
5	2.3	3.5	4	4	4	4
15	2.7	2.5	2.5	2.5	2.5	2.5
	Amount of foam in culture†					
5	3	3	3	3	3	3
15	3	3	1	0	0	0

Culture medium: Standard salts, $\frac{1}{2}$ strength; corn steep, 40 ml; lactose, 27.5 g; and distilled water to make 1 L.

* The figure 4 represents a deep yellow; lower numerals represent correspondingly less.

† Height of foam in inches above liquid level.

penicillin yields were obtained from cultures inoculated with the smaller quantity of pellets, and these cultures also produced a greater amount of yellow pigment and a more persistent head of foam. In these small submerged cultures the appearance of the yellow pigment and a persistent head of foam 3 to 4 cm in height has always been associated with good yields of penicillin.

Penicillin yields and changes in culture appearance which result from a slightly modified pellet medium are shown in table 9. A maximum penicillin yield of 112 units of penicillin per ml was obtained in 6 days. The initial pH of such a medium with 1 to 1.5 g of CaCO_3 per culture is 5.2 to 5.3, and without CaCO_3 , it is

4.0 to 4.2. Under these culture conditions (without CaCO_3), the pH rises from 4.0 to 4.2 up to 7.7 to 7.8 in 3 days. From the third to the fifth day there is a decrease in the pH, followed by a rise on the sixth and seventh days.

A penicillin yield of 41 units per ml has been obtained with a pellet inoculum in a medium containing a 2 per cent concentration of glucose, standard salts, 40 ml of corn steep liquor per liter, and 1.5 g CaCO_3 per culture. Other experiments showed that when the initial glucose concentration was raised much above 2 per cent, the pH rose too slowly and too heavy mycelial growth occurred. If less than 2 per cent of glucose was employed, the amount of growth was too light and the pH rose rapidly to about 8.2.

A series of cultures containing CaCO_3 was prepared with low initial glucose concentration. Different amounts of glucose were added daily, beginning on the

TABLE 9

Penicillin production and culture appearance with a pellet inoculum in submerged cultures of P. notatum NRRL 832*

	MEASUREMENTS MADE ON SUCCESSIVE DAYS					
	2	3	4	5	6	7
Units per ml.....	12	32	58	90	112	106
pH of medium.....	7.3	7.7	7.5	7.2	7.8	8.0
Formation of yellow pigment in medium.....	1.7	2.3	3.5	3.7	4.0	4.2
Average pellet diameter, mm.....	1.6	2.5	2.5	2.5	2.5	2.5
Amount of foam in culture.....	2	3	3	3	3	2

Medium for pellet inoculum: Corn steep liquor, 50 ml; standard salts; lactose, 10 g; glucose, 4.0 g; zinc sulfate, 0.044 g; and distilled water to make 1 L. Inoculated with a spore suspension, and 1.3 g of CaCO_3 per culture.

Production medium: Corn steep liquor, 40 ml; lactose, 27.5 g; glucose, 2.0 g; $\frac{1}{2}$ strength of standard salts; and distilled water to make 1 L. No CaCO_3 added to these cultures.

* 7.5 ml of a 3-day-old pellet inoculum per each production culture.

second day and ending on the sixth day. Penicillin yields and pH changes of the media are shown in table 10. The initial medium contained only 0.5 per cent lactose; hence, glucose was the major carbon source. The daily addition of 0.5 to 1.0 g of glucose to these cultures gave maximum penicillin yields of 72 units per ml. It seems possible that better penicillin yields may be obtained with glucose as the major carbon source if changes are made in the initial concentration of the carbon supply, and if a different schedule of nutrient additions is employed.

The best culture medium for penicillin production by the submerged growth of *P. notatum* NRRL 832 in shake flask cultures contained lactose and corn steep liquor in about one half the concentration found optimum by Moyer and Coghill (1945) for penicillin production in surface cultures of *P. notatum*, NRRL 1249.B21. An increase in nutrients above the optimal levels gave either an unfavorable pH change or a marked increase in fungus growth.

It has not been possible to determine the optimum pH for penicillin accumulation in these submerged cultures, although it is known that the pH increases during the course of the fermentation. Penicillin accumulation, however, appears to be optimum at higher pH levels than in the surface cultures with *P. notatum* 1249.B21. Penicillin potency usually drops rapidly when the pH of the medium increases above 8.0 to 8.2.

The pH change in cultures containing CaCO_3 and 4 per cent of corn steep liquor, inoculated with ungerminated spores (table 3), and in pellet-inoculated cultures without CaCO_3 (table 9), follows an interesting course. During the first

TABLE 10

Effect of adding glucose during the fermentation, with pellet-inoculated cultures low in initial carbon

	MEASUREMENTS ON SUCCESSIVE DAYS				
	3	4	5	6	7
	Oxford units per ml				
Feed 1.....	16	26	36	31	22
Feed 2.....	29	35	54	72	62
Feed 3.....	29	33	40	61	73
Feed 4.....	19	22	37	45	54
	pH of media				
	3	4	5	6	7
	pH of media				
Feed 1.....	7.85	7.9	7.95	8.1	8.4
Feed 2.....	7.75	7.7	7.75	7.95	8.0
Feed 3.....	7.6	7.55	7.6	7.7	7.65
Feed 4.....	7.0	6.8	7.2	7.6	7.5

Culture medium: Commercial glucose, 11 g; lactose, 5.5 g; $\frac{1}{2}$ strength of standard salts; corn steep, 30 ml; and distilled water to make 1 L. CaCO_3 , 1.0 g per culture. Ten ml of pellet inoculum per culture.

Feed 1, 5 ml containing no glucose.

Feed 2, 5 ml containing 0.5 g glucose.

Feed 3, 5 ml containing 1.0 g glucose.

Feed 4, 5 ml containing 1.5 g glucose.

First feed at 2 days and last feed at 6 days.

2 or 3 days of growth, there is a rapid rise, followed by a slight drop for 2 to 3 days, after which there is a final rise. In these cultures the carbohydrates present in the corn steep liquor support the early growth, after which there is a comparatively slow utilization of the lactose.

The same factors which influence the pH changes in the surface cultures (Moyer and Coghill, 1945) are believed to function in the submerged cultures. The speed of the pH change can be controlled to a large measure by adding glucose during the fermentation. This pH control is believed to be associated with the prevention of carbohydrate starvation, rather than with the production of an organic acid.

The role of corn steep liquor with respect to penicillin production in surface

cultures has been considered by Moyer and Coghill (1945). It is believed that the function of corn steep liquor is substantially the same in both surface and submerged fermentations. It has not yet been established that there are specific compounds in this medium which are required for penicillin synthesis by the fungus. The addition of corn steep liquor to suitable culture media enables the fungus to make a rapid growth, with pH changes which permit the accumulation of penicillin.

The superiority of lactose over glucose, sorbitol, and glycerol for penicillin production appears to be associated with the greater speed at which the latter are assimilated by the fungus. Starch is slowly hydrolyzed but has not been satisfactory for good penicillin production under submerged conditions. There is sufficient carbohydrate with glucose at 3.0 g per liter, in addition to the lactic acid and other reducing substances in the corn steep liquor, to permit the fungus to utilize the lactose. In such cultures, the fact that lactose is not so readily assimilated provides a more favorable pH change and a longer growth period. A rapid and dense fungus growth develops when the more readily assimilated carbon sources are supplied in concentrations corresponding to the optimal concentration of lactose. Because of the heaviness of this growth, and its effect on the motion within the flask, it is possible that these cultures received inadequate aeration. There is also an unfavorable pH change and a shorter period of active growth with these readily assimilated carbon sources. Glucose, as the major carbon source, will give good penicillin yields only if the initial concentration is low and if more glucose is gradually supplied during the course of the fermentation.

The addition of nutrients during the course of the fermentation has given increased penicillin yields. This culture feeding has given some pH control and has prolonged the productive life of the culture. These results suggest the feasibility of commercial application. The time of harvest is not so critical as in the case of cultures which are not fed. The increase in penicillin yield may, under an improved feeding schedule, more than compensate for the cost of the extra time required for the fermentation. The withdrawal of liquor from the partly completed fermentation, and the addition of more nutrients, suggest the possibility of a semicontinuous process.

SUMMARY

It was found that a strain of *Penicillium notatum*, NRRL 832, not related to the Fleming strain, gave higher penicillin yields than any other fungus tested in submerged culture. This strain has been widely used in the industrial production of penicillin in tanks.

The best medium for penicillin production contained lactose and corn steep liquor in about one-half the concentration found optimal for surface culture production.

Penicillin yields of 112 Oxford units per ml have been obtained in 6 days by using a pregerminated inoculum. Some increase in penicillin yields has been obtained by adding nutrients during the course of the fermentation.

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THE COMBINED ACTION OF PENICILLIN AND THE SULFONAMIDES IN VITRO: THE NATURE OF THE REACTION¹

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The combined action of penicillin and the sulfonamides against bacteria has been demonstrated by several workers. Ungar (1943) reported that penicillin and sulfapyridine showed a marked additive effect both *in vitro* and *in vivo* against *Staphylococcus aureus* and hemolytic streptococci. Soo-Hoo and Schnitzer (1944) reported the additive effect of penicillin when combined with sulfanilamide or sulfapyridine against streptococci. Kirby (1944) using sulfadiazine and Bigger (1944) using sulfathiazole found that these compounds when combined with penicillin showed an additive effect against staphylococci and streptococci. T'ung (1944) found the *in vitro* sulfathiazole-penicillin combination more efficient than either compound alone when tested against several strains of *Brucella*. Hobby and Dawson (1944a) found a slight additive effect with sulfapyridine and none with sulfadiazine when these agents were combined with penicillin and tested against staphylococci and streptococci. They also reported that the rate of penicillin activity was reduced when small amounts of the sulfonamides were added.

In the present paper we will report on the ability of three sulfonamides (sulfathiazole, sulfadiazine, and sulfapyrazine) to increase the activity of penicillin against ten sulfonamide-susceptible and sulfonamide-resistant strains of *S. aureus* and eight gram-negative rods. Observations indicating the mechanism of the *in vitro* additive effect are included in our experimental data.

MATERIALS AND METHODS

Media. Since extract and infusion broth markedly inhibited the action of the sulfonamides, a casein hydrolyzate medium (SMACO) was used, prepared according to the procedure of Strauss, Dingle, and Finland (1941). In preliminary titrations 20 per cent horse serum was added to the casein hydrolyzate medium, and, though this resulted in a slight reduction in titer, the results paralleled the data obtained with the unsupplemented casein hydrolyzate medium. The casein hydrolyzate medium, pH 7.2, was therefore used throughout the work.

Chemotherapeutic agents. Crystalline penicillin,² containing 1,667 units per mg, and commercial penicillin (Merck), containing 462 units per mg, were used.

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²The crystalline penicillin was received from the Committee on Medical Research of the Office of Scientific Research and Development through the courtesy of Dr. Chester S. Keefer.

No difference was observed in the inhibitory action of equivalent units of these two preparations. Stock solutions were prepared by diluting the penicillin in phosphate buffer, pH 7.2, to 100 units per ml and storing at 4 C for a maximum of two weeks, during which time there was no significant drop in titer. The stock solution was diluted in the casein hydrolyzate medium to the final desired concentration.

Sodium sulfathiazole and sodium sulfadiazine were prepared as 5 per cent stock solutions in phosphate buffer, pH 7.2, and stored at 4 C for a maximum of 2 weeks. They were diluted in the culture medium to the final desired concentration. Sulfapyrazine was diluted directly in the casein hydrolyzate medium.

Test organisms. Ten sulfonamide-susceptible and sulfonamide-resistant strains of *S. aureus* were kindly given us by Dr. M. G. Sevag. Five of the strains were sulfonamide-susceptible. The sulfonamide-resistant form of each of the strains had been obtained by growing the organisms in increasing concentrations of sulfathiazole (Sevag and Green, 1944).

The gram-negative organisms were *Escherichia coli*, *Eberthella typhosa*, *Aerobacter aerogenes*, *Salmonella schottmuelleri*, *Salmonella aertrycke*, *Salmonella enteritidis*, *Salmonella paratyphi*, and *Shigella paradysenteriae*. The bacteria were grown at 37 C and an 18- to 20-hour culture was diluted in phosphate buffer, pH 7.2, to the equivalent of 10 on the Klett-Summerson colorimeter (approximately 20,000,000 cells per ml). One-tenth ml of a suspension of the staphylococci and 0.2 ml of a suspension of the gram-negative rods were used as our inocula. The larger inoculum of the gram-negative organisms was used because of their greater susceptibility to the sulfonamides.

Penicillinase production. For our studies on the mechanism of the additive effect penicillinase production was determined by a method similar to that used by Bondi and Dietz (1944). The cultures of the organisms being tested for penicillinase were grown in casein hydrolyzate medium for 4 days at 37 C. To 1.5 ml of the supernatant fluid was added 0.1 ml of a solution of penicillin. Control solutions consisted of 1.5 ml of medium plus 0.1 ml of a similar concentration of penicillin. After remaining at room temperature for 1 hour the solutions were placed in glass cups (7 mm in inside diameter) which had been previously placed on agar plates seeded with *S. aureus*. A measure of the penicillinase production was obtained by comparing the zone of inhibition obtained with the penicillin plus the culture supernatant and the zone of inhibition obtained with the medium and penicillin.

Test procedure. Each organism was tested initially for its susceptibility to the sulfonamides and penicillin. The sulfonamide titrations were performed as follows: Dilutions of sulfonamides were prepared in 10 ml of medium and seeded with the standard inoculum. The cultures were incubated at 37 C; readings were made at intervals of 24, 48, and 72 hours, compared with the untreated controls, and recorded on the basis of visual turbidity as 0 (no growth), trace, 1, 2, 3, and 4. Penicillin titrations were performed in a similar fashion.

After determining the range of susceptibility of each organism for the sulfonamides and penicillin separately, the combined action of the two compounds was

determined. A penicillin titration was performed in parallel with a penicillin titration to which varying dilutions of the sulfonamides were added. A comparison of the penicillin titer with and without added sulfonamide was a measure of the additive action of the compounds. All eighteen organisms were tested against the sulfathiazole-penicillin combination. Only five organisms were tested against sulfadiazine and sulfapyrazine combined with penicillin since the results in the case of all three sulfonamides were found to be essentially similar.

The 48-hour turbidity readings were used as the basis of our titers since these readings were more consistent than the 24-hour titers. Ten penicillin titrations performed over a period of months on a single *S. aureus* (4a) strain showed that though the 24-hour readings of penicillin titrations varied between 0.025 and 0.25 of a unit, the 48-hour readings varied only between titers of 0.1 and 0.25 of a unit.

TABLE 1

Combined action of penicillin and sulfathiazole against sulfonamide-susceptible *Staphylococcus aureus* (5A)

UNIT OF PENICILLIN	SULFATHIAZOLE											
	A 1:10,000			B 1:25,000			C 1:50,000			D		
	Hours											
	24	48	72	24	48	72	24	48	72	24	48	72
0.1	0	0	0	0	0	0	0	0	0	0	0	0
0.075	0	0	0	0	0	0	0	0	0	0	0	0
0.05	0	0	0	0	0	1	0	tr	1	0	1	2
0.025	0	0	0	0	tr	1	0	1	2	0	2	2
0.01	tr	1	1	tr	2	3	2	3	4	2	2	3
0.005	2	2	3	2	3	4	3	4	4	3	4	4
0.001	2	3	4	3	4	4	4	4	4	4	4	4
Bacterial control.....										4	4	4
Sulfathiazole control.....	3	4	4	3	4	4	4	4	4			

RESULTS

Though, as will be indicated later, all our results may be explained on the basis of a single mechanism, we have separated our data into three groups. We have determined the action of sulfathiazole and penicillin against (a) the sulfonamide-susceptible staphylococci, (b) the sulfonamide-resistant staphylococci, and (c) the gram-negative rods. In table 1 a typical protocol is shown of the results obtained with sulfathiazole and penicillin against a sulfonamide-susceptible strain of *S. aureus* (5a).

The readings obtained at 24, 48, and 72 hours with penicillin alone are recorded in column D, and the readings of penicillin to which sulfathiazole was added in dilutions of 1:10,000, 1:25,000, and 1:50,000 are recorded in columns A, B, and C. The control readings at the bottom of column D indicate the growth of the bacteria in the absence of any inhibitor. The control readings at the bottoms of

columns A, B, and C represent the degree of inhibition obtained with the sulfathiazole alone. In column C, in which the sulfathiazole itself showed no inhibitory action, there was no evidence of an additive effect. We have found in all our titrations that if the sulfonamides or penicillin was used in concentrations that were in themselves not inhibitory no additive effect resulted. Sulfathiazole in a dilution of 1:10,000 had a slight inhibitory effect and markedly increased the titer of the penicillin. A 1:10,000 dilution is a convenient basis of comparison since it approximates the blood levels obtained with the sulfonamides. The increase in the penicillin titer on the basis of the 48-hour readings is fivefold. The remaining four sulfonamide-susceptible staphylococci when tested against the sulfathiazole-penicillin combination showed a twofold to fivefold increase in their susceptibility to penicillin in the presence of 1:10,000 sulfathiazole. In

TABLE 2

Combined action of penicillin and sulfathiazole against a sulfonamide-resistant Staphylococcus aureus (8A)

UNIT OF PENICILLIN	SULFATHIAZOLE											
	A 1:1,000			B 1:5,000			C 1:10,000			D		
	Hours											
	24	48	72	24	48	72	24	48	72	24	48	72
0.25	0	0	0	0	0	0	0	0	0	0	0	0
0.1	0	0	0	0	3	4	0	4	4	0	4	4
0.075	0	0	0	tr	4	4	1	4	4	1	4	4
0.05	0	0	4	tr	4	4	1	4	4	1	4	4
0.025	2	3	4	2	4	4	2	4	4	2	4	4
0.01	3	4	4	4	4	4	4	4	4	3	4	4
0.005	3	4	4	4	4	4	4	4	4	4	4	4
0.001	3	4	4	4	4	4	4	4	4	4	4	4
Bacterial control										4	4	4
Sulfathiazole control	3	4	4	4	4	4	4	4	4			

table 1 a comparison of the 24- and 48-hour readings obtained with the penicillin (column D) revealed a sharp reduction in the 48-hour titer, whereas the readings obtained with the 1:10,000 sulfathiazole and penicillin (column A) remained relatively constant over the 48-hour period. We have observed this difference in nearly all our titrations. The additive effect was most striking in those cultures showing a markedly delayed growth in the penicillin titrations. As will be indicated later, this difference in the 48-hour readings of the penicillin titrations when compared with the penicillin-sulfonamide combination is of importance in the interpretation of the additive effect.

Sulfonamide-resistant staphylococci. A typical protocol of the results obtained with the five sulfonamide-resistant strains of *S. aureus* is shown in table 2. The sulfonamide-resistant staphylococci were not inhibited by a 1:10,000 dilution of sulfathiazole, and there was no additive effect at this concentration with penicillin.

An increase in the sulfathiazole concentration to 1:1,000, which was in itself slightly inhibitory, did increase the penicillin titer, and the results approximated those obtained with a 1:10,000 dilution of sulfathiazole, as recorded in table 1 for the sulfonamide-susceptible staphylococci.

We found that there was no correlation between the sulfonamide resistance or susceptibility of the ten strains of staphylococci and their susceptibility to penicillin. The absence of any correlation between sulfonamide resistance and penicillin resistance of staphylococci has been demonstrated by Spink, Ferris, and Vivino (1944).

Gram-negative rods. Because of the greater resistance of the gram-negative rods to penicillin and their greater susceptibility to the sulfonamides, the dilutions of the various chemotherapeutic agents were modified accordingly. The results obtained with *S. paradyenteriae*, which are representative of those obtained with the gram-negative rods, are shown in table 3.

TABLE 3

Combined action of penicillin and sulfathiazole against Shigella paradyenteriae (Flexner)

UNIT OF PENICILLIN	SULFATHIAZOLE								
	A 1:250,000			B 1:300,000			C		
	Hours								
	24	48	72	24	48	72	24	48	72
10	tr	1	1	tr	1	1	3	4	4
1	1	2	3	1	2	4	4	4	4
0.75	1	2	3	1	2	4	4	4	4
Bacterial control.....							4	4	4
Sulfathiazole control.....	1	2	3	1	2	4			

S. paradyenteriae was highly susceptible to the action of sulfathiazole, though inhibition in 24 hours was incomplete in the concentrations used. Penicillin itself had but a slight inhibitory effect in concentrations of 10 units per ml. There was evidence of an additive effect when 10 units of penicillin were combined with sulfathiazole, though the effect was small. One unit of penicillin, which approximates the penicillin titer currently used, was in itself inactive and did not show any increase in titer when combined with sulfathiazole. The results obtained with *S. paradyenteriae* were representative of those obtained with *S. paratyphi*, *E. typhosa*, *S. aertrycke*, *S. enteritidis*, and *E. coli*, though the concentrations of sulfathiazole used for these gram-negative rods varied between 1:100,000 and 1:200,000 because of the greater resistance of these organisms to sulfathiazole. An additive effect was evident at concentrations of 10 units of penicillin per ml that was in itself slightly inhibitory at this concentration. However, *A. aerogenes*, which was completely resistant to 10 units of penicillin, showed no evidence of any additive effect in the presence of sulfathiazole. We found that our strain of *A. aerogenes* produced a large amount of penicillinase, which fact was probably related to its complete resistance to penicillin.

The most susceptible of the gram-negative rods was *S. schottmuelleri*. This strain was almost completely inhibited by 0.75 units of penicillin. At this concentration of penicillin there was a marked additive effect with 1:100,000 sulfathiazole that resulted in a fivefold increase in penicillin activity. In general, because of the relative inactivity of penicillin against the gram-negative rods, there was no marked evidence of an additive effect.

Sulfadiazine and sulfapyrazine. Two strains of sulfathiazole-susceptible staphylococci, two strains of sulfathiazole-resistant staphylococci, and *E. typhosa* were tested against the sulfadiazine-penicillin and sulfapyrazine-penicillin combinations. The sulfathiazole-resistant and sulfathiazole-susceptible staphylococci were resistant and susceptible, respectively, to the sulfadiazine and sulfapyrazine, and the magnitude of the additive effect was related to this resistance and susceptibility. *E. typhosa* was inactivated by sulfapyrazine and sulfadiazine in high dilution, and the additive effect obtained paralleled the results obtained with sulfathiazole.

Mechanism of the additive action of sulfonamides and penicillin. As was indicated previously, the inhibition of the organisms in 24 hours by certain concentrations of penicillin was usually followed in 48 hours by a rapid increase in the number of cells. In the presence of added sulfonamide this rapid increase was not observed.

In considering the mechanism of the additive effect obtained with the sulfonamides and penicillin the authors thought that the sulfonamides might act by preventing the production of penicillinase by the bacteria. Since certain bacteria are known to produce penicillinase during their growth, the reduction in the titer of penicillin during continued incubation might have resulted from the gradual inhibition of penicillin by penicillinase, and the presence of sulfonamide might have specifically inhibited the production of penicillinase. This was not found to be the case. Our ten strains of *S. aureus* were tested for penicillinase, and none of the strains were found to produce significant amounts of penicillinase, though a sharp reduction in the 48-hour penicillin titer was usually observed with these strains (tables 1 and 2). In addition, *A. aerogenes*, which we found to be an active penicillinase producer, showed no specific decrease in penicillinase production when grown in the presence of small amounts of sulfathiazole. The observed reduction in penicillinase production of *A. aerogenes* was related to the reduction in the number of viable cells.

It was thought that the increased growth in penicillin after 48 hours of incubation might be due to the lability of penicillin at 37 C. A standard penicillin titration with *S. aureus* was set up in quadruplicate. One series of tubes was seeded immediately and incubated at 37 C with the uninoculated tubes. After 24, 48, and 72 hours' incubation, respectively, each series of tubes was seeded with the standard inoculum. Titers were read for each series of tubes after 24, 48, and 72 hours. It was found that there was no detectable destruction of penicillin in those tubes which had remained 48 hours at 37 C prior to inoculation, whereas the 72-hour titration showed a very slight drop in titer. These results show that the concentration of penicillin remained essentially constant throughout the test period.

It seemed, therefore, that the delayed growth occurring in the presence of penicillin might be due to the growth of penicillin-resistant organisms and the additive effect might be related to the inhibition of penicillin-resistant organisms by the added sulfonamide. Cultures were obtained from penicillin titrations against *S. aureus* (4A) by subculturing after 48 hours' incubation organisms from those tubes which had shown no growth in 24 hours. Six subcultures were obtained, and the penicillin titers of these cultures were compared with the penicillin titers obtained with the original inoculum. The results of two typical titrations are shown in table 4.

In columns A and B are shown the results obtained with the subcultures. Column C gives the results of a titration run against the original inoculum of *S. aureus* (4A). The results show that there was approximately a tenfold increase in the penicillin resistance of the subcultures.

TABLE 4

Penicillin resistance of subcultures of Staphylococcus aureus (4A) obtained from penicillin titrations

UNIT OF PENICILLIN	48-HOUR SUBCULTURES						ORIGINAL INOCULUM		
	A			B			C		
	Hours								
	24	48	72	24	48	72	24	48	72
1.0	0	2	2	0	1	2	0	0	0
0.75	tr	3	3	0	1	2	0	0	0
0.5	1	3	3	1	1	2	0	0	0
0.25	2	3	4	1	1	2	0	0	3
0.1	3	4	4	2	2	4	0	4	4
0.075	3	4	4	2	3	4	0	4	4
0.05	3	4	4	3	4	4	tr	4	4
0.025	4	4	4	4	4	4	4	4	4
0.01	4	4	4	4	4	4	4	4	4
Bacterial control	4	4	4	4	4	4	4	4	4

An experiment demonstrating the ability of sulfonamides to inhibit the residual penicillin-resistant organisms is shown in table 5. Cell counts were made after 8 hours' incubation at 37 C on a sulfonamide-susceptible strain of *S. aureus* (4A), and the results were compared with the turbidity readings obtained at 24 and 48 hours. In the presence of concentrations of penicillin which did not completely inhibit (C, E), the readings at 8 hours were lower than those obtained with the penicillin-sulfathiazole combination (D, F). However, the organisms in the penicillin tubes, though showing a relatively small count in 8 hours, grew in 24 hours, and these organisms showed an increased resistance to penicillin. The cultures containing the sulfathiazole were able to inhibit these penicillin-resistant cells and showed inhibition of growth in 24 and 48 hours.

The slightly lower count obtained in 8 hours with penicillin alone is in keeping with the findings of Hobby and Dawson (1944a) that there is a slight reduction in the rate of penicillin activity when the compound is added simultaneously with

the sulfonamides to a culture. We have observed a more striking reduction in the rate of penicillin action when the compound was added 5 hours *after* the sulfathiazole (table 6). At the time the penicillin was added both the 5-hour cultures containing the 1:10,000 sulfathiazole and the control cultures were slightly turbid. Readings taken 16 hours after the penicillin was added showed that the cultures containing the sulfathiazole and penicillin were still turbid,

TABLE 5

Rate of action of sulfathiazole and penicillin on Staphylococcus aureus (4A)

	0 HOURS	8 HOURS	24 HOURS	48 HOURS
	Count		Turbidity	
(A) Organism control.	2,000,000	100,000,000	4	4
(B) 1:10,000 sulfathiazole....	2,000,000	70,000,000	2	2
(C) 0.05 unit of penicillin.	2,000,000	4,000,000	1	4
(D) 0.05 unit of penicillin and 1:10,000 sulfathiazole.	2,000,000	12,000,000	0	0
(E) 0.025 unit of penicillin.	2,000,000	20,000,000	3	4
(F) 0.025 unit of penicillin and 1:10,000 sulfathiazole.	2,000,000	22,000,000	0	0

TABLE 6

Action of penicillin on Staphylococcus aureus (4A) when added 5 hours after
1:10,000 sulfathiazole*

UNIT OF PENICILLIN	16 HOURS		48 HOURS		72 HOURS	
	Sulfathiazole and penicillin	Penicillin	Sulfathiazole and penicillin	Penicillin	Sulfathiazole and penicillin	Penicillin
0.25	1	0	0	1	0	2
0.1	1	0	0	2	0	2
0.075	1	0	tr	2	tr	3
0.05	1	tr	tr	2	tr	3
0.025	1	2	1	3	2	3
0.01	2	3	2	3	3	3
Bacterial control.	4	4	4	4	4	4
Sulfathiazole control....	2		3		4	

* At the time of the addition of the penicillin both the sulfathiazole and the control cultures were slightly turbid.

whereas the penicillin control tubes had cleared. However, in 48 hours the sulfathiazole-penicillin cultures showed a marked clearing, and the penicillin controls were turbid. The 48-hour titers were then found to be essentially similar to those obtained when both agents were added simultaneously.

These results may be explained in the following fashion: Penicillin was actively bactericidal and lytic when the culture was actively multiplying. When the penicillin was added 5 hours after the sulfathiazole, the sulfathiazole had

passed the lag period in its activity and inhibited the rapid multiplication of the bacteria. In the absence of active bacterial growth penicillin activity was inhibited. Since the 1:10,000 sulfathiazole was not completely inhibitory, the bacteria were still capable of multiplication, and the delayed destruction of the bacteria by the penicillin then occurred. In the control tubes the active multiplication of the organisms permitted the prompt action of the penicillin with the usual delayed growth of the resistant cells occurring in 48 hours. When penicillin and sulfathiazole were added simultaneously, as in table 5, there was a lag in the penicillin activity, though this lag was relatively slight. Under these conditions the penicillin could exert its inhibitory action during the active multiplication of the organisms and before the sulfonamide became inhibitory. This inhibition of penicillin activity in the presence of a bacteriostatic agent has previously been observed by Chain and Duthie (1945) in their studies on the combined action of penicillin and helvolic acid. We have found that when the penicillin was added 5 hours before the sulfathiazole the results were similar to those obtained when both agents were added simultaneously.

That the resistance of bacteria to penicillin and sulfonamides varies independently has been reported by Spink, Ferris, and Vivino (1944) and also observed by us in the study of our ten strains of *S. aureus*. It has also been established that there is an inverse relationship between the size of the bacterial inoculum and the sulfonamide titer. Henry (1943) cites an extensive series of references on this point. For example, we have found 1:10,000 sulfathiazole only slightly inhibitory when seeded with the standard inoculum of *S. aureus*. However, the 1:10,000 sulfathiazole became completely inhibitory when the inoculum was diluted 1:10. These observations permit the following explanation of the combined action of penicillin and sulfonamides.

Penicillin in concentrations which do not completely inhibit growth reduces the number of viable bacteria with the most susceptible organisms being destroyed first. A certain number of the more resistant organisms remain viable and ultimately grow. However, in the presence of added sulfonamide the penicillin reduces the number of viable bacteria to the point at which the added sulfonamide completely inhibits growth (table 5). If the penicillin is not present in sufficient concentration to reduce the number of bacteria significantly (e.g., *A. aerogenes*) or if the sulfonamide is not present in inhibitory concentrations (e.g., 1:10,000 sulfathiazole acting on sulfonamide-resistant *S. aureus*, table 2), no additive effect will result. The report of Hobby and Dawson (1944b) that sulfadiazine did not result in an additive effect when combined with penicillin was probably due to the very small concentration of sulfadiazine used, which was in itself not inhibitory.

DISCUSSION

We have not observed any *in vitro* evidence of antagonism between the sulfonamides and penicillin other than the delay in the rate of penicillin activity when added 5 hours after the sulfathiazole. Both agents appear to inhibit independently. This delay in penicillin activity in the presence of sulfathiazole may be

of some clinical importance, particularly when the penicillin is acting at the site of an infection only for a brief period. Where the penicillin therapy is sustained, the delay in action may not be of importance since, as we have shown, this initial delay is soon overcome.

We have not considered the combined action of penicillin and the sulfonamides in mixed infections. Under such conditions the sulfonamides might be extremely effective by destroying penicillinase-producing bacteria.

It may be of interest to mention that though the sulfonamides are reported as being ineffective in the treatment of typhoid fever, the high degree of susceptibility of *E. typhosa* to the sulfonamides that we have observed suggests that they may be effective prophylactically. Hardy (1943) has reported that sulfadiazine has a marked bacteriostatic action against *E. typhosa* in the enteric tract, though the organisms usually returned after cessation of therapy. This would indicate that though the sulfonamides cannot remove the bacteria from certain foci of infection they can destroy bacteria that are free in the bowel. It would appear possible that in an epidemic the sulfonamides might play a prophylactic role against the spread of typhoid fever. The problem of sulfonamide-resistant strains of *E. typhosa* developing under such conditions would not be of major importance since the compounds are not therapeutically effective.

SUMMARY

Ten sulfonamide-resistant and sulfonamide-susceptible strains of *Staphylococcus aureus* and eight gram-negative rods were tested for their susceptibility to the combined action of penicillin and sulfonamides.

The *in vitro* combination of sulfathiazole, sulfadiazine, or sulfapyrazine and penicillin resulted in an increase in the penicillin titer only if both agents were present in inhibitory concentrations.

A marked delay in penicillin activity was observed when this compound was added to a bacterial culture 5 hours after the sulfathiazole. No other *in vitro* evidence of antagonism between the sulfonamides and penicillin was observed.

The additive effect of penicillin and sulfonamide inhibitions is interpreted as a result of the reduction by penicillin of the total number of bacterial cells to limits within which the sulfonamide becomes completely inhibitory.

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TWO PARACOLON CULTURES RELATED ANTIGENICALLY TO *SHIGELLA PARADYSENTERIAE*

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In recent years numerous reports have been made on the antigenic interrelationship of organisms of the *Salmonella*, *Escherichia*, and *Shigella* genera. (Bornstein *et al.*, 1941; Saphra and Silberberg, 1942; Wheeler *et al.*, 1943; Edwards, Cherry, Bruner, 1943). Stuart and coworkers (1943) have described the antigenic factors shared in common by *Shigella alkalescens*, paracolon, and coliform organisms isolated from a small population in a restricted area. Recently Saphra and Wassermann (1945) have demonstrated that *Salmonella onderstepoort* and *Escherichia coli* 391 have an identical "O" antigen, while *Shigella paradysenteriae* (Flexner "V") is related to the other two by an antigen fraction.

The present report deals with two paracolon strains, isolated from the same patient, which are related to various Flexner and Boyd types of *Shigella paradysenteriae*.

Isolation of the paracolon strains was made by one of us from the stools of a 7-month-old infant with a past history of eczema. Immediately following the addition of egg yolk to his diet, the child began to vomit and a few hours later to have a diarrhea. There was no previous history of diarrhea in the patient, nor among contacts. The diarrhea subsided within a few hours. The paracolon denoted 339 in the following description was obtained from a diarrheal specimen; the second paracolon strain, 848, was obtained from a formed specimen approximately 14 days after the original isolation. In blood serum taken shortly after the second paracolon was isolated, no agglutinins were present for either organism.

Both strains upon initial isolation gave a dysenterylike reaction on Kligler's iron agar slants because of delayed gas production. It is customary in the laboratory in which the isolations were made to test all dysenterylike organisms by the slide agglutination technique with absorbed sera covering the genus *Shigella* and the specific types of the *S. paradysenteriae* complex. The sera are of commercial origin. A marked agglutination reaction was obtained in only one of the sera, Boyd P 143. Both 339 and 848 organisms were also agglutinated strongly in an unabsorbed polyvalent Flexner antiserum used for tube agglutination tests. From the preliminary evidence it was natural to conclude that the organisms were *Shigella paradysenteriae*. Biochemical tests revealed, however, that both organisms were of the paracolon group with nearly identical characteristics. The biochemical characteristics are listed in table 1.

An antiserum was developed in a rabbit against paracolon 339 that agglutinated both 339 and 848 organisms in a dilution of 1:20,480. Absorption of this

antiserum with 848 organisms revealed that agglutinins for both strains were removed completely. An antiserum was also prepared against 848 paracolon that agglutinated the two strains in a 1:20,480 dilution. Agglutinins for both 339 and 848 organisms were completely removed by absorption with 339 organisms. The paracolon strains were, therefore, antigenically similar.

In tables 2 and 3 are recorded the results of agglutination tests with 339 and 848 sera and various *Shigella* cultures. Slightly different results were obtained with the two sera; both, however, agglutinated suspensions of Flexner "V" "X," and "Y" organisms in moderate titer. Neither serum in low dilution agglutinated suspensions of P 143 organisms.

The reaction of 339 and 848 organisms with absorbed P 143 antiserum was interesting from a diagnostic standpoint. In order to discover whether antigenic

TABLE 1
Characteristics of paracolon strains

	STRAIN 339	STRAIN 848
Glucose.....	AG 24 hours	AG 24 hours
Lactose.....	AG 144 hours	A 120 hours
Sucrose.....	AG 72 hours	AG 72 hours
Maltose.....	AG 24 hours	AG 24 hours
Mannitol.....	AG 24 hours	AG 24 hours
Sorbitol.....	AG 24 hours	AG 24 hours
Salicin.....	no reaction	no reaction
Dulcitol.....	AG 96 hours	A 168 hours
Indole.....	produced	produced
Urea.....	no reaction	no reaction
Acetylmethylcarbinol.....	not produced	not produced
H ₂ S.....	not produced	not produced
Citrate.....	utilized 72 hours	utilized 72 hours
Motility.....	sluggish at 37 C	none at 37 C or 22 C

AG = acid and gas.

A = acid.

relationships existed with other enteric organisms, suspensions of both cultures were tested with a number of unabsorbed *Shigella* antisera and with 25 *Salmonella* "O" antisera. No agglutinations were obtained with the *Salmonella* sera.

In table 4 are recorded the reactions of 339 and 848 paracolon strains with unabsorbed *Shigella* antisera. Both strains were agglutinated in practically the same dilutions of serum; the results are, therefore, incorporated in one table. Flexner "Y" antiserum agglutinated 339 and 848 in a dilution of 1:1,280, indicating a fairly strong antigenic bond. Others of the Flexner and Boyd antisera, including Boyd P 143, agglutinated the organisms in low dilutions ranging from 1:80 to 1:320.

When the paracolon strains were tested with type-specific paradysentery antisera of our manufacture, prepared according to Wheeler (1944), no reactions occurred in Flexner "V," "W," "Z," Boyd 103, Boyd P 119, or Boyd 88 antisera.

TABLE 2
Reaction of Shigella strains with paracolon 339 antiserum

LIVE ANTIGENS	SERUM DILUTION								SALINE CON- TROL
	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
<i>S. paradyenteriae</i>									
Flexner "V"	4	4	4	3	—	—	—	—	—
Flexner "W"	—	—	—	—	—	—	—	—	—
Flexner "X"	4	4	4	4	2	—	—	—	—
Flexner "Z"	4	3	1	—	—	—	—	—	—
Flexner "Y"	4	4	4	4	2	—	—	—	—
Boyd 103	—	—	—	—	—	—	—	—	—
Boyd P 119	—	—	—	—	—	—	—	—	—
Boyd 88	—	—	—	—	—	—	—	—	—
Boyd 170	—	—	—	—	—	—	—	—	—
Boyd P 288	—	—	—	—	—	—	—	—	—
Boyd D 1	—	—	—	—	—	—	—	—	—
Boyd D 19	—	—	—	—	—	—	—	—	—
Boyd P 274	—	—	—	—	—	—	—	—	—
Boyd P 143	—	—	—	—	—	—	—	—	—
<i>S. alkalescens</i>	—	—	—	—	—	—	—	—	—
<i>S. sonnei</i>	—	—	—	—	—	—	—	—	—
<i>S. ambigua</i>	—	—	—	—	—	—	—	—	—
<i>S. dysenteriae</i>	—	—	—	—	—	—	—	—	—

Incubation, 52 C, water bath, 18 hours.

4 = complete agglutination.

— = no reaction.

TABLE 3
Reaction of Shigella strains with paracolon 848 antiserum

LIVE ANTIGENS	SERUM DILUTION								SALINE CON- TROL
	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
<i>S. paradyenteriae</i>									
Flexner "V"	4	4	4	4	4	2	—	—	—
Flexner "W"	2	1	—	—	—	—	—	—	—
Flexner "X"	4	4	4	4	4	2	—	—	—
Flexner "Z"	4	4	±	—	—	—	—	—	—
Flexner "Y"	4	4	4	4	4	4	2	±	—
Boyd 103	2	2	1	—	—	—	—	—	—
Boyd P 119	4	2	—	—	—	—	—	—	—
Boyd 88	4	2	—	—	—	—	—	—	—
Boyd 170	—	—	—	—	—	—	—	—	—
Boyd P 288	—	—	—	—	—	—	—	—	—
Boyd D 1	—	—	—	—	—	—	—	—	—
Boyd D 19	—	—	—	—	—	—	—	—	—
Boyd P 274	—	—	—	—	—	—	—	—	—
Boyd P 143	—	—	—	—	—	—	—	—	—
<i>S. alkalescens</i>	—	—	—	—	—	—	—	—	—
<i>S. sonnei</i>	—	—	—	—	—	—	—	—	—
<i>S. ambigua</i>	—	—	—	—	—	—	—	—	—
<i>S. dysenteriae</i>	—	—	—	—	—	—	—	—	—

Incubation, 52 C, water bath, 18 hours.

4 = complete agglutination.

— = no reaction.

Since strains of Flexner "X" and "Y" are regarded by Boyd (1936, 1938) and Wheeler (1944) as possessing no type-specific antigens, it is evident that the relationship of the paracolon strains 339 and 848 with the Flexner group¹ is a sharing of minor antigens.

During the preparation of a new absorbed P 143 antiserum for use in slide agglutination tests, it was discovered that agglutinins for the paracolon strains 339 and 848 were not removed by repeated absorption with three Flexner "X" strains and two strains of Flexner "Y." Agglutinins for all other *Shigella*, with

TABLE 4
Reaction of paracolon strains 339 and 848 with Shigella antisera, unabsorbed

SERA	DILUTIONS OF SERA								
	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
<i>S. paradyenteriae</i>									
Flexner "V".....	4	1	—	—	—	—	—	—	—
Flexner "W".....	4	3	2	—	—	—	—	—	—
Flexner "X".....	4	4	±	—	—	—	—	—	—
Flexner "Z".....	4	4	4	2	—	—	—	—	—
Flexner "Y".....	4	4	4	4	4	2	±	—	—
Boyd 103.....	—	—	—	—	—	—	—	—	—
Boyd P 119.....	4	4	3	—	—	—	—	—	—
Boyd 88.....	4	4	2	±	—	—	—	—	—
Boyd 170.....	3	—	—	—	—	—	—	—	—
Boyd P 288.....	4	4	2	—	—	—	—	—	—
Boyd D 1.....	±	—	—	—	—	—	—	—	—
Boyd D 19.....	±	—	—	—	—	—	—	—	—
Boyd P 274.....	4	3	±	—	—	—	—	—	—
Boyd P 143.....	4	4	4	2	—	—	—	—	—
<i>S. alcalescens</i>	—	—	—	—	—	—	—	—	—
<i>S. sonnei</i>	—	—	—	—	—	—	—	—	—
<i>S. ambigua</i>	—	—	—	—	—	—	—	—	—
<i>S. dysenteriae</i>	—	—	—	—	—	—	—	—	—

Incubation, 52 C, water bath, 18 hours.

Paracolon strains were in the "S" phase and were not agglutinated by saline.

4 = complete agglutination.

— = no reaction.

the exception of Boyd P 143, were removed. Further absorption of this antiserum with 339 and 848 organisms did not remove specific agglutinins for Boyd P 143, although complete removal of agglutinins for the paracolon strains was effected.

In order to rule out the possibility that the reaction of 339 and 848 with both absorbed and unabsorbed Boyd P 143 antisera was due to "natural" agglutinins, a new antiserum was prepared against Boyd P 143 organisms.² The rabbit to be

¹ The terminology of Boyd (1940) is used here to denote types Flexner V, Z, W, X, Y, Boyd 103, Boyd P 119, and Boyd 88.

² Source of culture: Dr. C. A. Peluffo, Instituto de Higiene, Montevideo.

immunized was tested before inoculation and found to have no agglutinins for either 339 or 848. Following immunization, the new Boyd P 143 antiserum agglutinated the homologous strain in a dilution of 1:5,120; it agglutinated the paracolon strains in a dilution of 1:640.

It is evident that a Boyd P 143 antiserum prepared by inoculation of the strain used by us will, if absorbed with *Shigella* strains only, contain agglutinins for the paracolon strains 339 and 848.

DISCUSSION

The foregoing description of the antigenic relationship of paracolon strains 339 and 848 with types of *Shigella paradyserteriae* is additional evidence of the lack of sharp antigenic demarcation between organisms of the Enterobacteriaceae. How commonly paracolon strains of the type described may be encountered can only be conjectured. It is probable the occurrence is rare. The chief interest to the medical bacteriologist in these organisms lies in the fact that their antigenic relationship with Boyd P 143 led to an error in the rapid slide agglutination test.

We do not believe that antigenic relationships such as those described in this paper invalidate the use of absorbed *Shigella* antisera for rapid identification by slide technique. Our experience with such sera, together with biochemical confirmation tests, leads us to believe that the percentage of error is very low.

The lack of reciprocal agglutinins in the paracolon antisera for Boyd P 143 organisms is not an unusual finding. Berger (1945) has described a similar phenomenon in his analysis of a new antigenic type of *Shigella paradyserteriae*, differing only in degree from the relationship described here. It appears from our experience in producing an absorbed Boyd P 143 antiserum that the agglutinins present for 339 and 848 strains of paracolon are distinct from the type-specific fraction and are not removed by absorption with other *Shigella* strains which are agglutinated by this serum in low dilution.

SUMMARY

Two paracolon strains, isolated from the same patient, that bear identical antigens and that share minor antigens with the Flexner group of *Shigella paradyserteriae* are described.

In addition, a nonreciprocal relationship of Boyd P 143 with the paracolon strains is described.

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ON THE EXISTENCE, MORPHOLOGY, NATURE, AND FUNCTIONS OF THE CYTOPLASMIC MEMBRANE IN THE BACTERIAL CELL

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Until a few years ago knowledge of the superficial structures of the bacterial cell was in a state of confusion, not only in terminology but also in concept (see the review by Lewis, 1941). In 1930 the author demonstrated simultaneously, by a simple procedure, a differentiated outer layer of the cytoplasm, which he then called "ectoplasm," and a cell wall. In view of the confusing terminology, the author later (Knaysi, 1938) wrote: "Morphologically, the bacterial cell is surrounded by three membranes which may be called: the *cytoplasmic membrane*, the *cell wall*, and the *slime layer*." This statement was confirmed by all subsequent observations (Knaysi, 1941, 1944), and the existence of three membranes was accepted by Mudd *et al.* (1941, 1944), Lewis (1941), and Dubos (1945).

Of the three membranes, the cytoplasmic membrane is probably the most important, and yet it is the one the existence, nature, and properties of which require further consideration. This structure was seen, but usually misinterpreted, by several investigators. Among these was Migula (1897) who, following Bütschli (1890), called it the "Wandbelag" and considered it to be the entire cytoplasm. According to Migula, the cavity limited by this "Wandbelag" is occupied by a relatively huge vacuole, and not by a *central body* as Bütschli believed. Migula's vacuole is what we now consider the cytoplasm proper, in which endospores are formed and vacuoles may appear; for if Migula's concept were correct, we would have to postulate the existence of vacuoles of the second order. Eisenberg (1909) recognized the cytoplasmic membrane as the "Rindenschicht," which together with the membrane (our cell wall) constitute the "Ektoplasma." Arthur Meyer (1912) did not recognize any structure which corresponds to the cytoplasmic membrane. He wrote at considerable length of the membrane (our cell wall), but it is evident from his description of its reactions that he, at times, was inadvertently describing the cytoplasmic membrane. He thus attributes to his membrane a high refractive power (p. 151, and figure 34) and states that in bacteria which deposit fat the membrane contains fat (p. 149 and figure 27 of his plate I).

The difficulty in the way of a satisfactory proof for the existence of the cytoplasmic membrane is in the fact that over much of the evidence heretofore presented hangs the shadow of the possibility that it is due to a surface phenomenon (e.g., Henrici, 1934, pp. 122-123). The same difficulties would have been true for the demonstration of the cell wall were it not for the fact



FIG. 1. *Bacillus megatherium*, C₁. From a 5 hour old culture at 33 C, on a slant of the medium $\overline{\text{MITG}}_2 + 1.5 \text{ g}$ agar. Stained by Knaysi's (1941) cell wall method. Early stage of autolysis.

FIG. 2. *Bacillus megatherium*, C₁. Age, medium, and treatment as in figure 1. A ghost cell in which the cytoplasm has completely disappeared but the cytoplasmic membrane is still intact.

FIG. 3. *Bacillus megatherium*, C₁. Age, medium, and treatment as in figure 1. A ghost cell in which the cytoplasm has completely disappeared and the cytoplasmic membrane is partially autolyzed.

FIG. 4. *Bacillus megatherium*, C₁. Age, medium, and treatment as in figure 1. A ghost cell of which only the cell wall and an inclusion remain.

that shrinkage of the cytoplasm leaves the cell wall isolated and makes its demonstration more certain. Consequently, if it were possible to separate the cytoplasmic membrane from the cytoplasm, the evidence for its existence would immediately take the dimensions of proof. This was accomplished by making use of the process of autolysis.

In cultures of *Bacillus megatherium* and of *Bacillus cereus* on agar slant media, particularly in the presence of glucose, numerous ghost cells appear like empty cell walls. However, by using the author's method (Knaysi, 1941), one finds that in different ghost cells autolysis has reached different stages. In some cells, the cytoplasm has disappeared, leaving the cytoplasmic membrane, sometimes intact, still lining the inner surface of the cell wall (figure 2). In more advanced stages of autolysis, fragments of the cytoplasmic membrane may still be seen here and there at the inner surface of the cell wall (figure 3). The cytoplasmic membrane appears dark red, and the cell wall blue. Upon further autolysis, the cell wall takes only a faint purplish color (figure 4). Sometimes the cell wall may be seen surrounded by a bright red layer of variable thickness, the slime layer (figure 5).

Investigation of ghost cells accomplishes two purposes: first, it separates the cytoplasm from its membrane, thus isolating the latter; second, it shows that the cytoplasm is much more susceptible to autolysis than the cytoplasmic membrane and, consequently, that the two structures are widely different in chemical composition or physicochemical structure.

Morphology and Properties of the Cytoplasmic membrane

In previous reports (Knaysi, 1938) it was stated that the cytoplasmic membrane is a superficial layer of the cytoplasm characterized by a relatively high refringence and affinity for dyes, that it is the principal (not the only) substrate for the gram and acid-fast stains, retaining the dye after the cytoplasm has been

FIG. 5. *Bacillus megatherium*, C₁. Age, medium, and treatment as in figure 1. The cell wall and the slime layer.

FIG. 6. *Bacillus cereus*, C₂. A microculture 4 hours and 45 minutes old at 27 C. Medium as in figure 1. Cells living *in situ* showing cytoplasmic membrane and its extensions, which are potential places of division. Note the early plane form of these extensions.

FIG. 7. *Bacillus cereus*, C₁. The first two generations of an endospore; the exine is still visible. Microculture prepared by mixing 13-hour-old culture at 33 C in VFC* + 0.1 g of K-phosphate mixture (initial pH = 6.8), with an equal volume of $\frac{\text{MITG}^1}{4}$, and a loopful of this mixture planted on a droplet of 1.5 per cent bacto agar. Age of microculture 2 hours at 28.5 C. It shows uneven thickness of cytoplasmic membrane and two stages of cell division.

FIG. 8. *Bacillus cereus*, C₁. From a microculture prepared as in figure 7. When the microculture was 1 day old at 27 C, it was disconnected and the cover glass dropped in hot, 10 per cent glucose solution for the Sharp test. Note positive cytoplasmic membrane and granules of the two sporangia.

FIG. 9. *Bacillus cereus*, C₂. One-day-old microculture at 30 C prepared as in figure 7. It shows accumulation of cytoplasmic membrane material at a potential place of division.

FIG. 10. *Bacillus cereus*, C₁. One-day-old microculture at 30 C prepared as in figure 7. The cover glass was removed and subjected to the Feulgen procedure (Knaysi, 1942); hydrolysis 10 minutes at 60 C. Note positive membrane and granules.

¹MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose.

²VFC = 100 ml of vitamin-free casein hydrolyzate.

completely decolorized, and that it contains lipoids and proteins at least partly in chemical combination. We have also described the important role the cytoplasmic membrane plays in the division of the bacterial cell (Knaysi, 1929, 1938, 1941, 1944); indeed, it is the most conspicuous structure during that process, and in this respect the bacterial cell is different from the yeast cell (Knaysi, 1941); this difference constitutes a criterion by which dividing yeasts can be easily distinguished from large bacteria. We have also reported (1944) the surprising observation that the cytoplasmic membrane gives a positive Feulgen reaction.

During the present investigation we have repeated and confirmed most of the observations given above. Very careful study, in bright field, of bacteria in microcultures revealed that the inner surface of the cytoplasmic membrane is usually not a smooth curve but is jagged or wavy, even in actively growing cells (figures 6, 7, 12), and that the inner protoplasm of a single cell is not usually a perfectly homogeneous mass but is frequently segmented by thin, plane films originating from the cytoplasmic membrane and identical with it in physical and chemical properties (figures 6, 12). Some of these films are, potentially, places at which the cell ultimately divides (figures 6, 7). Whether these films are identical with those postulated by Dubos (1945) we are unable to say.

The *thickness* of the cytoplasmic membrane is variable even in the same cell (see above, also figures 6, 7, 11, 12). It is logical, however, to consider as normal thickness that observed in cells, or substantial portions of cells, where fluctuations are at a minimum. Measurements at various stages in the development, from germ cells to sporangia, of *Bacillus cereus*, strain C₃, gave the range of 0.21 to 0.35 μ . In view of the fact that the cytoplasmic membrane completely surrounds the cytoplasm, the total thickness of the membrane amounts to 0.3 to 0.4 of the total thickness of the protoplasm (i.e., exclusive of the cell wall). Under certain conditions the membrane extension across the cell, previous to division, may be abnormally thick (figure 9). After division, part of this material remains at each of the contiguous ends of the sister cells as "polar bodies" or "polar caps" which, on account of their behavior toward dyes, etc., have often been mistaken for nuclei.

The *structure* of the cytoplasmic membrane is not always homogeneous. At the time of cell division one observes the formation of a granule at the place where the centripetal growth of the membrane is to take place and a thinning of the membrane immediately beyond that point. It seems as if the granule is the result of the accumulation of the substance of the membrane. In several members of the genus *Bacillus* there is a stage of cultural development, normally preceding that of sporulation, in which the membrane of the living cell becomes heterogeneous throughout (figure 11); this heterogeneity is due to the formation of granules which can be discharged into the cytoplasm under aerobic conditions.

Chemically, the cytoplasmic membrane gives the Sharp test for protein (figure 8), stains well with the dyes of the Sudan series (figure 13), and gives a positive Feulgen reaction (figure 10); these characteristics survive autolysis of the cytoplasm and treatment with fat solvents; the membrane has an isoelectric range

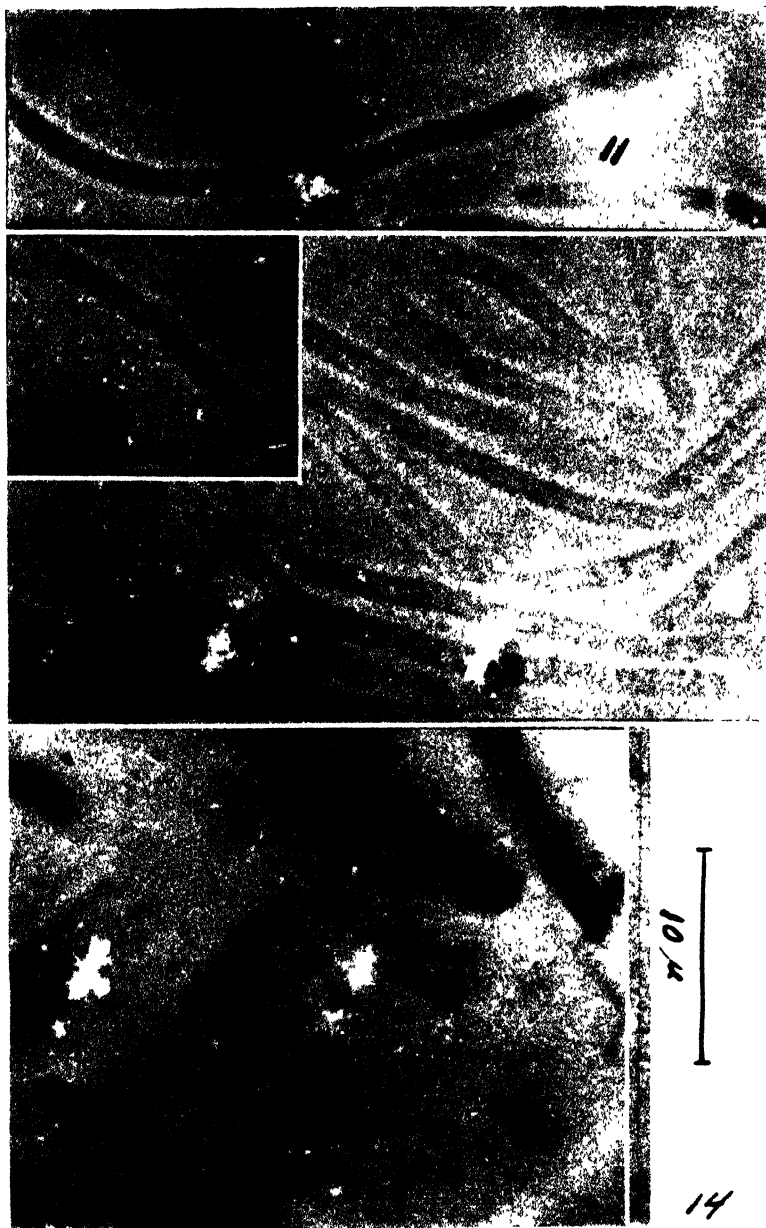


FIG. 11 *Bacillus cereus*, C₃. Cells from one-day old microculture on $\frac{\text{MITG}^1}{2}$ at 28 C.

It shows heterogeneity of cytoplasmic membrane.

FIG. 12 *Bacillus cereus*, C₃. A few hour-old microculture at 29 C, prepared by placing a loopful of an 18 hour old culture in VFC² + 0.1 g K-phosphate mixture (initial pH = 6.8) on a droplet of bacto agar. It shows uneven thickness of the cytoplasmic membrane and the lamellalike extensions of the membrane which divide the cytoplasm into chambers.

FIG. 13 *Bacillus megatherium*, C₁. Cells from a 7-hour old slant culture at 33 C on the medium $\frac{\text{MITG}^1}{2}$ + 1.5 g agar, mounted in Sudan black B. It shows the cytoplasmic membrane and its cross extensions.

FIG. 14. Scale for all illustrations

much below that of simple proteins, even in gram-negative species. We are, therefore, justified in stating that at least the *principal* component of the cytoplasmic membrane, in gram-positive as well as in gram-negative species, is a highly stable combination of lipoids and proteins, namely, lipoprotein. There is also evidence (see under Discussion and Conclusions) that this lipoprotein molecule may be conjugated with some complex organic radical.

Function of the Cytoplasmic Membrane

At the present time two roles are definitely known to be played by the cytoplasmic membrane. The most conspicuous role is in cell division; the other is in permeability (Knaysi, 1944). Very recently (1945), we observed the formation of lipoprotein granules by the cytoplasmic membrane in a few aerobic spore-formers, and the elimination of these granules into the cytoplasm before sporulation. Having been unable to ascertain the function of these granules, we cannot give this process the proper interpretation.

DISCUSSION AND CONCLUSIONS

The greatest obstacle to a satisfactory demonstration of the cytoplasmic membrane has been the objection that both accumulation of dyes in the surface layer of the cytoplasm and high refringence of that layer may be merely surface phenomena. Admittedly, this objection has been difficult to answer, although one may be convinced, from long and intimate observations of the bacterial cell, that there is more to the cytoplasmic membrane than a mere optical or physicochemical artifact. The author had always considered the possibility that the cytoplasmic membrane, although real, might owe its origin to an accumulation of surface-active material. Recently (1944), however, he presented evidence indicating that, although this hypothesis may be true from the point of view of evolutionary differentiation, this differentiation has already become established in the bacterial cells with which he is familiar. Indeed, in the cells of many common species investigated, the cytoplasmic membrane is in no way a passive structure which merely accumulates on surfaces. It is a permanent, active, even, so to speak, aggressive structure which initiates, rather than follows, the division of the cell; for if the formation of the cytoplasmic membrane followed the division (by constriction) of the cytoplasm, the newly formed membranes would have curved surfaces, normal to the long axis of the cell. Actually, the centripetal growth of the cytoplasmic membrane which initiates cell division is, at first, in a plane normal or *oblique* to that axis (figures 6, 12) and curves only as it splits, thus separating the sister cells.

Several years ago (see Knaysi, 1944, p. 35) we made the observation that the cytoplasmic membrane of both gram-positive and gram-negative bacteria gives a positive Feulgen reaction. This means that at least one component of the cytoplasmic membrane contains free, or potentially free, aldehyde groups, and that this component is not removed by hydrolysis with normal HCl for 10 minutes at 60 C. In view of what we know about the chemical make-up of the

membrane and the discovery by Claude (1939) of a lipid which reacts with Schiff's reagent, the interpretation seems to be obvious. It is necessary, however, to discuss the possibility of the following two hypotheses:

The first is indicated by the observation of Dubos and MacLeod (1938) that treatment of the heat-killed, gram-positive cells of pneumococci with a polynucleotidase obtained from animal tissues renders those cells gram-negative. Henry and Stacy (1943) secured the same result by the action of certain carbohydrate-splitting enzymes or by extraction with a solution of bile. The extraction of gram-positive cells with a 2 per cent aqueous solution of bile removes some carbohydrate and a substance identified as Mg-ribonucleate, leaving a gram-negative skeleton rich in basic protein material. If this skeleton is treated with formaldehyde (or certain other reducing agents) and soaked in the extract, the Mg-ribonucleate recombines with the skeleton and the cells become gram-positive again. Henry and Stacy believe that they have been able to show "that the essential constituent in Gram-positive organisms. . . lies in their having as part of their surface structure the magnesium salt of ribonucleic acid." In view of the fact established by the author (first reported in 1938, p. 94) that the *principal* substrate of the gram and acid-fast reaction is the cytoplasmic membrane, this membrane must be the "surface structure" mentioned by Henry and Stacy. The possibility of the presence of a ribonucleate radical in the lipoprotein complex of the cytoplasmic membrane is in no way contradictory to cytological data and had been suspected by the author (1929) when he wrote: "The membrane (of the tuberculosis organism) stains metachromatically with old methylene blue solutions," and when he further concluded that the membrane and granules of that organism "may be considered as a peculiar combination of fat with protein and other materials." Metachromatism with old solutions of methylene blue is usually considered characteristic of nucleic acids. Ribonucleic acid, free or as nucleoprotein, is known to be gram-positive (Delétang, 1933; Knaysi, 1943) and to react with Schiff's reagent (Knaysi, 1942). Consequently, it would be expected to give a positive Feulgen reaction if it is not removed in the process of hydrolysis; and it is conceivable that, granting its presence in the cytoplasmic membrane, it may be in a stable combination which resists acid hydrolysis as carried out in the Feulgen technique. However, one has also to postulate that it exists in the cytoplasmic membrane of the gram-negative bacteria in a form which resists hydrolysis by acid but which is removed in the gram technique, or that it is so bound that its acidic radicals are not totally free.

The second hypothesis that, in strains without differentiated nuclei, the cytoplasmic membrane contains the nuclear material of the cell at first appears attractive in view of the conspicuousness of the membrane in cell division. However, when one considers that the endospore, which contains nuclear material, is formed free in the cytoplasm, without any apparent co-operation from the cytoplasmic membrane of the mother cell, the soundness of such a hypothesis is questioned.

SUMMARY

The study of spontaneously autolyzed cells in cultures of *Bacillus cereus* and *Bacillus megatherium* made possible the isolation and demonstration of the cytoplasmic membrane. The fact that this membrane is much more resistant to autolysis than the cytoplasm proper indicates considerable chemical or physico-chemical differences between the two structures. The cytoplasmic membrane stains with dyes of the Sudan series and gives the Sharp test for proteins and a positive Feulgen reaction. It consists principally of lipoids and proteins in a highly stable chemical combination. The internal surface of the cytoplasmic membrane is jagged and wavy; besides surrounding the cytoplasm, it forms plane films which separate the cells into compartments and which are potential places of cell division; it forms and eliminates into the cytoplasm granules similar to itself in chemical composition; its demonstrated roles are in cell division and in permeability. The thickness of the cytoplasmic membrane varies even in a single cell; in young cells of *Bacillus cereus* it is usually in the range 0.21 to 0.35 μ . The significance of the membrane's positive Feulgen reaction is discussed.

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NOTE

A NEW SALMONELLA TYPE: SALMONELLA CUBANA

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This culture was recently sent to us by Dr. Arturo Curbelo of Havana who had isolated it in an outbreak of diarrhea among baby chicks. Ten per cent of the animals had died. The organism was found in the feces and in one instance in the heart blood of the affected animals. The birds were imported from Florida and arrived apparently in good health in Cuba. They were fed on a synthetic "starter" food. Ten days later the epizootic started. No case of human infection was reported.

The culture consisted of gram-negative, motile rods with the cultural characteristics of the *Salmonella* group. Glucose, mannitol, and maltose were acidified with gas formation. Sorbitol, xylose, dulcitol, inositol, rhamnose, arabinose, and trehalose were promptly fermented. H₂S was formed. Lactose, sucrose, and salicin were not attacked; gelatin was not liquefied within 15 days. Indole was not produced. On feeding, the culture was not pathogenic for mice, but killed them within 20 hours when injected subcutaneously.

The strain was agglutinated by a *Salmonella* *worthington* O serum (I.XIII.XXIII) to its end titer, and by sera of *Salmonella* *poona* (XIII.XXII), *Salmonella* *paratyphi* A (I.II), *Salmonella* *senftenberg* (I.III.XIX), and *Salmonella* *onderstepoort* (I.VI.XIV.XXV) types to fractions of their homologous titers. All the other sera were negative. On the other hand, a serum prepared with boiled antigen of the new strain agglutinated the homologous culture as well as *S. worthington* to its end titer and gave cross reactions with the other types to a lesser degree. Mutual absorption tests proved the complete identity of the O antigen of this strain with that of *S. worthington*.

The flagellar antigen was agglutinated only by a z_{29} antiserum derived from *Salmonella* *tennessee* (VI.VII; z_{29} —Bruner and Edwards: Proc. Soc. Exptl. Biol. Med., 50, 174).

No second phase could be found by testing numerous single colonies or by the application of the Gard technique. The culture removed all H antibodies against *S. tennessee* from the z_{29} serum; this type in turn removed all H antibodies from a serum prepared with a formalized broth culture of the new strain. The H antigen is thereby confirmed as z_{29} . It is monophasic like that of *S. tennessee* and is also identical with the z_{29} of *Salmonella* *canastel*, a diphasic type recently described by Randall and Bruner (J. Bact., 49, 511). A transplant of the latter type which was given to us by Dr. Edwards of Lexington, Kentucky, was included in all agglutination and absorption tests.

It is perhaps worthy of note that of the five known members of the I.XIII.XXIII group two have been found in Cuba exclusively: *Salmonella havana* (Schiff and Saphra; J. Infectious Diseases, 68, 125), and the new type which will be named *Salmonella cubana*.

SUMMARY

S. cubana, a new *Salmonella* type isolated in Cuba from diseased baby chicks, has been described. It has the formula I.XIII.XXIII; z₂—.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND EIGHTY-SECOND MEETING,
PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING,
OCTOBER 23, 1945, PHILADELPHIA, PA.

BACTERIAL PENICILLINASE: PRODUCTION, NATURE, AND SIGNIFICANCE. *Amedeo Bondi, Jr., and Catherine Dietz*, Temple University School of Medicine, Philadelphia, Pa.

Bacteria which produce penicillinase, an enzyme which destroys penicillin, are widely distributed in nature. Although penicillinase-producing organisms are most frequently encountered in the coliform group and among the gram-positive spore-forming bacilli, members of the *Shigella* and *Mycobacterium* groups and approximately 14.0 per cent of *Staphylococcus* strains are also penicillinase producers. A medium containing a readily available source of organic nitrogen is essential for the production of an active preparation of penicillinase by a given organism. The presence of fermentable carbohydrate prevents active production due to destruction of the enzyme by the acid produced. Penicillinase is relatively heat-labile, being destroyed readily at 60 C for 30 minutes. Preparations from various organisms differ in their stability to heat, indicating that there may be more than one penicillinase. Activity is best retained by storage of preparations at freezing temperatures.

Inability of an organism to produce penicillinase is not an indication of an organism's susceptibility to penicillin. An organism which produces penicillinase is not likely to be very susceptible to penicillin. Of 115 strains of *Staphylococcus* studied, 16 or 13.9 per cent were not inhibited by penicillin in concentrations less than 0.45 units per ml. All 16 of these resistant strains produced penicillinase. The resistance of these *Staphylococcus* strains results from the destruction of penicillin by the enzyme as it is produced. This is indicated by the fact that small inocula of

such strains of *Staphylococcus* are inhibited by penicillin almost as readily as penicillin-susceptible organisms. Organisms made resistant to penicillin *in vitro* do not acquire the ability to produce penicillinase.

THE COMBINED ACTION OF PENICILLIN AND SULFONAMIDE IN VITRO: THE NATURE OF THE REACTION. *Morton Klein and Seymour S. Kaller*, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The combined *in vitro* inhibitory action of penicillin and three sulfonamides (sulfathiazole, sulfadiazine, and sulfapyrazine) was tested against 18 bacteria. Five were sulfonamide-susceptible strains of *Staphylococcus aureus*, five were sulfonamide-resistant *S. aureus*, and eight were gram-negative rods.

It was found that if both agents were present in inhibitory concentrations synergism resulted. If either one of the agents was not in itself inhibitory, no synergism resulted. The degree of synergism obtained was related to the concentration of the two agents and the resistance of the bacteria.

The synergistic action is explained as resulting from an initial reduction in the total number of bacteria by the penicillin, which permits a concentration of sulfonamide, which was only partially inhibitory in the presence of a large number of cells, to become completely inhibitory in the presence of a smaller number of cells. It was found that a delayed growth of *S. aureus* occurring in 48 hours in the penicillin control tubes was due to the development of penicillin-resistant bacteria. The inhibition of these penicillin-resistant forms by the added sulfonamide markedly increased the synergistic effect.

Sulfathiazole was found to effect a temporary but marked inhibition of penicillin

activity when the sulfonamide was present in the cultures five hours before the addition of the penicillin.

STUDIES ON BLOOD AGAR. I. AN IMPROVED BLOOD AGAR BASE. *Ezra P. Casman*,
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A simply prepared blood agar base has been developed which possesses the growth-supporting properties of bases prepared with fresh beef infusion. When used with candle jar incubation, it is possible to use one medium for the isolation of most of the pathogens (including *Neisseria gonorrhoeae*) and for the detection of group A hemolytic streptococci which give an alpha reaction on the surface of other blood agars. The presence of a small amount of niacinamide stimulates to a slight extent the growth of *Hemophilus influenzae*.

The formula for the blood agar base is:

Bacto proteose peptone no. 3... 1.0%
Bacto tryptose... 1.0%

Beef extract..... 0.3%
Sodium chloride..... 0.5%
Niacinamide..... 0.005%
p-Aminobenzoic acid 0.005%
Glucose..... 0.05%
Corn starch..... 0.1%
Washed agar..... 1.3-1.4%
Cold distilled water

Granular bacto agar is washed as a 0.4 or 0.5 per cent suspension in distilled water in the cold for five or six days, changing the water daily. The washed agar may be dehydrated with one or two volumes of 95 per cent alcohol before drying with moderate heat.

The mixture is stirred to disperse the starch, and dissolved by boiling. The pH is adjusted to 7.3 and the medium is autoclaved at 121 C for 15 minutes. On cooling to 45 to 50 C, 5 per cent human or horse blood and 0.0375 per cent water-lysed blood (0.15 per cent of a mixture of one part blood and three parts sterile distilled water) are added.

CONNECTICUT VALLEY BRANCH

ST. JOSEPH COLLEGE, WEST HARTFORD, CONNECTICUT, NOVEMBER 7, 1945

STUDIES ON THE SEROLOGY OF THE ESCHERICHIA COLI GROUP. *F. Kauffmann*, State Serum Institute, Copenhagen, Denmark.

Within the coli group it has been possible to establish a diagnostic schema, based on antigenic features. Pathogenic strains have been found frequently to be selective, serologically defined types. These pathogenic types have been recognized as important etiological factors in infections of the urinary and bile tracts, particularly, however, in appendicitis and secondary peritonitis. We can differentiate between two main groups of pathogenic coli types: (1) Hemolytic, necrotizing types which mostly belong to the O groups 2, 4, or 6. (2) The capsule-forming types belonging to the O groups 8 and 9, mostly nonhemolytic and nonnecrotizing.

The taxonomy of *Enterobacteriaceae* should start with the serological type diagnosis. Bergey's classification (as laid down in his Manual) is obsolete because it is based only on morphological and a few cultural features. An exact type diagnosis, however, is only possible by introducing

serological criteria. Bergey's schema in order to be useful would require a complete revision along serological lines.

We can only define correctly individual types, but not groups. The classification of individual types into one group or another will always be arbitrary. There are no sharply differentiated groups in nature, intermediary types are frequent, and our *Salmonella-Shigella*-coli classification (necessary as it may be for practical purposes) will always bear the stigma of an arbitrary expediency.

THE DIMINUTION IN THE NUMBER OF ORAL ORGANISMS FOLLOWING THE USE OF CARBAMIDE PEROXIDE IN GLYCEROL. *L. W. Slanetz*, Department of Bacteriology, University of New Hampshire, Durham, New Hampshire, and *E. A. Brown*, Department of Medicine, Tufts Medical School, Boston, Massachusetts.

The bactericidal effect of carbamide peroxide on the oral flora was determined by a combination of *in vivo* and *in vitro* tests. The carbamide peroxide consisted of a solu-

tion of 4 per cent carbamide peroxide in substantially anhydrous glycerol stabilized with 8-hydroxyquinoline (1:1,000). This solution, diluted with an equal volume of water, was used for washing the teeth and as a mouth rinse.

When samples from the mouth were plated in tryptone glucose extract agar after one application of the antiseptic solution, there was a reduction of from 26 to 93 per cent in the total number of bacteria normally present in the mouths of six different subjects. Samples plated on crystal violet potato extract agar and incubated anaerobically showed a reduction of 52 to 93 per cent in the number of fusiform bacteria. The lactobacilli appeared to be unaffected by the solution. Dark-field examination of scrapings from gingival crevices showed a marked germicidal action of the antiseptic for the oral spirochetes.

USE OF ANTIBIOTIC SUBSTANCES FOR FREEING TRICHOMONAS FETUS FROM BACTERIA.

L. F. Williams and W. N. Plastring, University of Connecticut, Storrs Agricultural Experiment Station, Department of Animal Diseases, Storrs, Connecticut.

The antagonistic action of bacteria of bovine origin for *Trichomonas fetus*, and its control by the use of antibiotic agents were investigated. The medium described by Plastring (J. Bact., 45, 196) was employed, with and without added antibiotic agents. The test mediums were inoculated with 1,000, 100, and 10 motile trichomonads per ml, and with test cultures of bacteria, and

were incubated at 37 C. Daily observations were made for bacterial growth and number of motile trichomonads per ml of culture.

In the absence of antibiotic agents the trichomonads were completely destroyed in less than 24 hours by *Streptococcus viridans*, *Streptococcus faecalis*, *Streptococcus lactis*, *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus vulgaris*, and in 48 hours by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Moderate growth of *T. fetus* occurred in the presence of *Micrococcus tetragenus*, *Sarcina lutea*, and *Bacillus subtilis*.

Clavacin, gramicidin, and actinomycin, when added to the basic medium in concentrations sufficient to control bacteria, were toxic for *T. fetus*. Penicillin in a concentration of 100 Oxford units per ml was not inhibitive for *T. fetus*, but destroyed the gram-positive bacteria used as contaminants. Streptomycin in a concentration of 100 units per ml destroyed all bacterial contaminants except *S. aureus* and *P. aeruginosa*. One thousand units per ml were required to free *T. fetus* from *P. aeruginosa*. The use of 100 units each of penicillin and streptomycin combined per ml of medium controlled all bacterial contaminants sufficiently to permit growth of *T. fetus*.

Tubes of the penicillin-streptomycin medium which were inoculated with the centrifugal sediment of artificially infected washings taken from the sheath of a healthy bull yielded pure cultures of *T. fetus* when the original material contained as few as one trichomonad per ml.

EASTERN NEW YORK BRANCH

ALBANY, NEW YORK, NOVEMBER 30, 1945

USE OF MOVING AVERAGES AND INTERPOLATION TO ESTIMATE MEDIAN EFFECTIVE DOSE. William R. Thompson, Division of Laboratories and Research, New York State Department of Health, Albany.

THE EFFECTS OF QUININE ON SAURIAN MALARIAL PARASITES. Paul E. Thompson, Mary Imogene Bassett Hospital, Coopers-town, New York.

Quinine was found to have an antimalarial action in cold-blooded animals, similar to

that described by other workers in warm-blooded animals. Observations were made on two species of malarial parasites in lizards.

Plasmodium floridense, the blood-induced infections of which do not include exoerythrocytic stages, responded readily to quinine therapy. Several doses of the drug eradicated the patent parasitemia. Evidence of a direct antiparasitic effect was observed in that the drug caused the parasites to become abnormal in their morphol-

ogy and staining reactions, and quinine reduced the mean number of merozoites per segmenter.

Infections of *Plasmodium mexicanum*, which produces numerous exoerythrocytic stages, were more refractory to treatment than were those of *P. floridense*, and this difference appeared to be due to the nonsusceptibility of the exoerythrocytic stages to quinine. The exoerythrocytic parasites included those of the *Plasmodium elongatum* type in virtually all blood and blood-forming cells, and those of the *Plasmodium gallinaceum* type in macrophages and true endothelial cells. Quinine exerted no discernible effect upon the number and appearance of either of these types of exoerythrocytic stages, but the parasites in erythrocytes were obviously affected by the drug.

LIMITING VALUES FOR ANTIBODY:ANTIGEN RATIO IN THE COMBINATION OF DIPHTHERIA TOXIN WITH SPLIT ANTITOXIC PSEUDOGLOBULIN. *Jaques Bourdillon*, Division of Laboratories and Research, New York State Department of Health, Albany.

In order to determine the composition of the split antitoxin-toxin complex, the re-

agents were mixed in various proportions and allowed to stand. The precipitate (which forms only around optimal ratio) was discarded, and the remaining fluid was titrated by flocculation to determine the amount of excess toxin or antitoxin left. When increasing amounts of antitoxin were added to constant amounts of toxin, at first the amount of complex formed was proportional to the antitoxin and decreasing amounts of toxin were left in excess, then both reagents were completely bound; finally, a limiting value was reached for the bound antitoxin:toxin ratio, and all additional antitoxin remained free. Plotting the amount of complex formed against antitoxin added thus yielded a curve composed of three straight segments, the last one horizontal. The same type of curve was obtained when the antitoxin was kept constant and the toxin varied. The limiting values for antitoxin:toxin molecular ratio were 1.2 (lower limit in toxin excess) and 3.8 (upper limit in antitoxin excess). These data and the mode of formation of split diphtheria antitoxin would not support the assumption that this antibody is multivalent.

CENTRAL NEW YORK BRANCH

GENEVA, NEW YORK, DECEMBER 1, 1945

"FOLIC ACID" REQUIREMENTS OF THE "MINUTE" STREPTOCOCCI. *C. F. Niven, Jr., Mary R. Washburn, and J. M. Sherman*, Laboratory of Bacteriology, Cornell University, Ithaca.

The nutritive requirements of the "minute" streptococci have been determined. In this work the 5 strains of group F and 10 strains of the "minute" variety of group G studied were found to require folic acid. The quantity needed by these organisms corresponds to the amount required by *Streptococcus faecalis* R, that is, approximately 3 millimicrograms per 10 ml for half-maximum growth.

As opposed to the reports for *S. faecalis* R and *Lactobacillus casei*, thymine could not replace folic acid for the "minute" streptococci. The SLR factor was also in-

active. One sample of the *L. casei* factor (from liver) showed variable activity toward the individual strains; 5 strains showed no response to this substance after 72 hours of incubation.

In addition to folic acid, all strains required nicotinic acid, pantothenic acid, riboflavin, and biotin. Thiamine and pyridoxine stimulated growth. An atmosphere containing approximately 10 per cent carbon dioxide was essential for growth in a chemically defined medium.

These organisms are the only streptococci known to require folic acid besides a small proportion of strains of the enterococci (group D). The "nonminute" variety of group G streptococci does not require folic acid.

THE ANTAGONISTIC ACTION OF THE BY-PRODUCTS OF A CULTURE OF *ASPERGILLIS WENTII* ON THE LEGUME BACTERIA. Robert S. Robison, Department of Agronomy, Cornell University, Ithaca.

A culture of *Aspergillus wentii* which was found to be antagonistic to strains of the legume bacteria was employed in these tests. When grown on an agar medium with strains of the legume bacteria isolated from *Lotus corniculatus*, *Vicia faba*, and *Medicago sativa* it produced zones of inhibition of 15 to 20 mm in diameter. The culture grew on a modified Czapek-Dox liquid medium and produced the active principle. Maximum activity against the strains of *Rhizobium* was noted after about ten days of incubation. The formation of the active principle appears to be correlated with the production of a yellowish-brown pigment. The active material can be adsorbed from the mother liquor by activated charcoal, eluted by certain strongly polar solvents, taken to dryness, dissolved in distilled water, pH 5.2, and still retain activity against the test strains of *Rhizobium*. The action against these strains appears to be primarily bacteriostatic. A second culture, *Aspergillus wentii* NRRL 375, did not produce the active substance.

Experiments conducted with growing legume plants in which the antagonistic culture of *Aspergillus wentii* was added to sterile and unsterile soils inoculated with the legume bacteria indicate that this fungus may reduce nitrogen fixation by red clover (*Trifolium repens*) since it reduced the numbers of nodules on the plants.

SPONTANEOUS DEVELOPMENT OF BUBBLES OF GAS IN AGAR MEDIUM. J. K. Wilson, Department of Agronomy, Cornell University, Ithaca.

A sterile agar medium developed bubbles spontaneously when brought from a storage temperature of 4 C to room temperature that was about 25 C. This medium contained 50 ml of yeast extract, 50 ml of an extract of asparagus, some nutrient salts, 5 g of Difco beef extract, 20 g sucrose, and 15 g agar in 1 L of water. The bubbles resembled those that may be produced by fermentation. Such bubbles were formed only when the beef extract was added. Lots of beef extract bearing 3 different control numbers were employed. If the yeast or the asparagus extract or both were omitted, gas appeared to develop less abundantly. Also the beef extract alone appeared to give only a few bubbles. No identification of the gas was attempted. It is assumed that the medium held a larger volume of gases at the lower temperature and that these were released when the medium was brought to a higher temperature, giving rise to the bubbles.

ANTIBIOTICS AND PERTUSSIS. Elizabeth Day and W. L. Bradford, University of Rochester, Rochester.

QUARTERNARY AMMONIUM SALTS AS GERMICIDES. R. F. Brooks and W. P. Van Eseltine, N. Y. State Agricultural Experiment Station, Geneva.

NODULATION TESTS WITH SPECIES OF *ASTRAGALUS*. Chi-han Chin, Department of Agronomy, Cornell University, Ithaca.

RELATIONSHIP OF THE GENUS *SERRATIA* TO THE COLIFORM AND COLIFORMLIKE ORGANISMS. Robert S. Breed, N. Y. State Agricultural Experiment Station, Geneva.

THE SIGNIFICANCE OF THE TYPE OF LACTIC ACID AMONG LACTIC ACID BACTERIA. Carl S. Pederson, N. Y. State Agricultural Experiment Station, Geneva.

THE NUTRITION OF PHYTOPATHOGENIC BACTERIA

I. MINIMAL NUTRITIVE REQUIREMENTS OF THE GENUS *XANTHOMONAS*

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Study of the nutrition of phytopathogenic bacteria has lagged far behind research in comparable aspects of animal pathogens. In recent years the nutrition of a few species has received adequate treatment; for the remainder of the species, however, knowledge of this subject is limited to not more than casual and uncritical observations of growth in certain of the synthetic media recommended by Smith (1905). (In this connection, see the general literature review in Lewis' work, 1930.) Unfortunately, many of these data are of little value today because, with the exception of some recent investigations, no attention was devoted to refinements in methodology which present knowledge deems indispensable. Consequently, glassware and media components were often not unquestionably free of contamination with active traces of growth-affecting substances, nor was there usually any apparent attempt to control the carry-over of similarly active trace nutrients in the inoculum.

This lack is all the more regrettable when one considers the values pertaining to a thorough understanding of the nutrition of plant-pathogenic bacteria. Parasitism involves a nutritional interrelationship, for during its pathogenetic existence the phytopathogen derives its entire nourishment from the host's tissues. Therefore, information concerning the sort of food which the bacterium can use *in vitro* might indicate what is taken by the pathogen from the host plant. Moreover, such data would permit evaluation of any relationship which might exist between nutritional requirements and the high degree of host specificity exhibited by many species. It would be well to keep in mind, also, the potential value to the bacterial taxonomist of knowledge concerning exact nutritive needs, especially since nutritive differences or similarities have proved significant taxonomic characters in other groups of microorganisms. In addition, one should not lose sight of the important advances in biochemistry (cf. van Niel, 1944) which are deriving from the current general interest in microbial nutrition.

With these ultimate aims in mind, a comprehensive, comparative study of the nutrition of phytopathogenic bacteria was undertaken. A preliminary survey has been published (Starr and Weiss, 1943) devoted to the utilization of asparagine as sole carbon and nitrogen source by the heterogeneous phytopathogenic

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² Preliminary experiments were conducted at the Haskins Laboratory, New York 17, New York. The writer is greatly indebted to the director and staff of that institution, and especially to Dr. S. H. Hutner, for generous assistance throughout the entire study.

bacteria formerly lumped in the genus *Phytomonas* Bergey *et al.*³ This work can be summarized by stating that, with a few exceptions, the green fluorescent *Pseudomonas* spp. and the tumor-inducing *Agrobacterium* spp. grow well upon serial transfer in a synthetic asparagine medium; whereas the yellow-pigmented, polarly flagellated, gram-negative *Xanthomonas* spp. and the gram-positive *Corynebacterium* spp. fail to grow. Subsequently, attention was focused on the nutrition of the genus *Xanthomonas* and of the phytopathogenic corynebacteria. The latter group will be the subject of a separate communication. The present discussion is limited to the minimal nutritive requirements of the genus *Xanthomonas*. To avoid misunderstanding it should be stated that the present study represents an attempt to obtain fair or good growth of *Xanthomonas* species in the simplest possible (i.e., minimal) media of known composition. It does not purpose to strive for the exact degree or rate of development which may be attained in complex media; merely a fair approximation of that ideal.

I

The validity of a comparative study is dependent to a great extent upon the authenticity of the cultures which are employed. In this connection the writer has been very fortunate in gaining the co-operation of Professor W. H. Burkholder, who very generously furnished the nucleus of the culture collection used in this and other studies. Additional authentic isolates were secured from various investigators; their assistance, also, is greatly appreciated.

The donor's identification of each culture was accepted if consistent with habitat and if the generic features matched those given for the genus *Xanthomonas* in the sixth edition of *Bergey's Manual of Determinative Bacteriology*.⁴

The purity of each culture was established by morphological examination and by repeated plating from dilute suspensions of cells. Particular care was necessary during plating to shake the cell suspensions thoroughly in order to insure separation of contaminants from the characteristic gummy *Xanthomonas* growth. Plating was repeated on various media and under different incubation conditions to provide every opportunity for contaminants to reveal themselves.

Inasmuch as the virulence of some phytopathogenic bacteria is lost or reduced during cultivation on laboratory media and this loss might be expected to have

³ The heterogeneous nature of *Phytomonas* Bergey *et al.* has been discussed by Burkholder (1930, 1939). Rahn (1929) and others have pointed out the "practical impossibilities" of using plant pathogenicity as a prime taxonomic character. With the recognition (cf. Elliott, 1937) that, contrary to a resolution of the Second International Congress for Microbiology, *Phytomonas* is used homonymously for a group of protozoan flagellates and for the aforementioned bacterial phytopathogens, the bacterial genus was dropped and its species placed in somewhat more suitable genera: *Agrobacterium* (Conn, 1942), *Corynebacterium* (Jensen, 1934; Dowson, 1942), *Pseudomonas*, *Xanthomonas* (Dowson, 1939), with a few species in the genus *Bacterium* in the sense used by Breed and Conn (1936). Much of the synonymy is tabulated by Weiss and Wood (1943) and will be incorporated in the forthcoming sixth edition of *Bergey's Manual of Determinative Bacteriology*.

⁴ The kindness of Professors R. S. Breed and W. H. Burkholder in making available the galley proof of the genus *Xanthomonas* in advance of publication, is gratefully acknowledged.

some influence on nutritive requirements, it was desirable to learn something about the pathogenicity of the cultures at the time they were used in the present study. Unfortunately, lack of facilities made it impossible to retest all the cultures, but through the co-operation of several plant pathologists,⁵ a number of the isolates were tested on their usual host plants as indicated in table 2. From the results obtained with the tested cultures, it is likely that most of the isolates used in this study were virulent.

Stock cultures were maintained on glucose potato agar at 12 C, or on glucose yeast-extract CaCO₃ agar at room temperature, and transferred every 7 to 9 weeks. Duplicates of most cultures were preserved successfully by lyophilizing in the Flosdorf-Mudd (1935) apparatus; some for as long as 5 years.

TABLE 1
Composition of the NH₄Cl, glucose, and salts basal medium

	g
Glucose (added separately)	0.5
NH ₄ Cl	0.1
KH ₂ PO ₄	0.2
MgSO ₄ ·7H ₂ O.	0.02
	μg
B[H ₃ BO ₃]	0.5
Ca[CaCO ₃]	10.0
Cu[CuSO ₄ ·5H ₂ O]	1.0
Fe[FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O]	10.0 or 50.0
I[KI]	0.1
Mn[MnSO ₄ ·H ₂ O]	1.0 or 2.0
Mo[MoO ₃]	1.0
Zn[ZnSO ₄ ·7H ₂ O]	5.0
Distilled water to 100 ml	
pH adjusted to 6.8 with NaOH	

II

The nutritive requirements of the cultures were determined by conventional means. At first, experiments were conducted in test tubes until it was observed that the growth of these aerobic organisms was being limited by an insufficient air supply (cf. Rahn and Richardson, 1940, 1942). Heavier growth occurred in shallow flask cultures or in continuously shaken test tube cultures. Instructive in this connection is a comparison of the turbidities developed by unshaken 10-ml cultures of various *Xanthomonas* species in 50-ml conical flasks (surface diameter 40 to 45 mm) and in upright test tubes (surface diameter 14 mm). Typical of the results are those obtained with *X. campestris* grown from small inocula in the basal medium which is described in table 1: after 7 days' incuba-

⁵ Thanks are due Drs. P. A. Ark, W. H. Burkholder, W. A. F. Hagborg, H. H. Thornberry, and J. R. Wallin for furnishing information regarding the virulence of these isolates.

tion at 28 C the average turbidities, expressed as "densities" (see below), of 10 replicate flask cultures was 0.25, that of 10 replicate tube cultures was 0.05, and that of the uninoculated medium was 0.

In view of the superiority of flask cultures for the purpose of the present study, in subsequent experiments 10-ml portions of media were distributed in 50-ml flasks. These were capped by suitable beakers or plugged with absorbent cotton. Cotton plugs were avoided, especially in experiments which might be affected by nitrilites introduced from the cotton (Sherwood and Singer, 1944); in general, it might be said that no difficulties attended the use of plugs made from absorbent, surgical cotton.

All glassware was cleaned by soaking in dichromate and sulfuric acid for not less than 24 hours, washing in tap water, and rinsing in distilled water. At times,

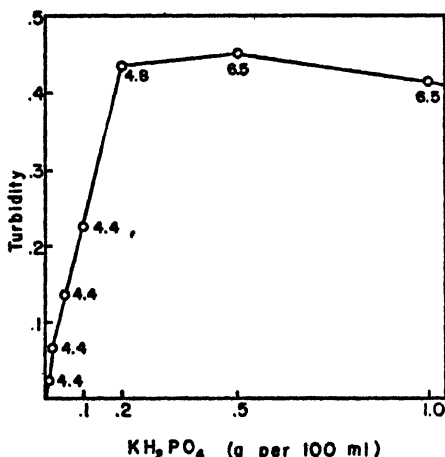


FIG. 1. EFFECT OF CONCENTRATION OF NEUTRALIZED KH_2PO_4 , ADDED TO THE PHOSPHATE-FREE BASAL MEDIUM, ON THE GROWTH OF *XANTHOMONAS CAMPESTRIS* ISOLATE XC1

Turbidity measured after 15 days' incubation and expressed in "density" units (see text). Initial hydrogen ion concentration was pH 6.8; final pH's are shown on the graph.

an alkaline cleaning mixture similar to that of Gaddis (1942) was used with equally satisfactory results.

The composition of the basal medium used in these experiments is shown in table 1. In practice, the inorganic components were dissolved in about half the necessary volume of water, supplemented by test nutrients if any, made up to volume, distributed in flasks, and autoclaved. Sterile glucose was then added aseptically in the form of a 25 or 50 per cent solution prepared by treating glucose solutions twice at pH 3.0 with norit charcoal, filtering each time with the aid of super-cel, and autoclaving the resulting solution (Hutner, 1944).

Inasmuch as scanty growth in early experiments was traced to an inadequate buffer, it would be appropriate at this point to mention an experiment which led to the choice of a more suitable phosphate concentration. The basal medium minus phosphate was supplemented with various concentrations of neutralized

KH_2PO_4 ; after autoclaving, glucose was added in the manner described above. Series of these media were inoculated uniformly with 10 *Xanthomonas* isolates which are able to grow in the basal medium, and, after incubation, turbidities and hydrogen ion concentrations were determined. The results, such as those graphed in figure 1, definitely point to a need for adequate buffer if a heavy growth of *Xanthomonas* is to be attained in simple media. Sufficient buffer is needed, too, if one is to avoid so-called "growth-stimulating" effects which are actually due to the buffer capacity of the nutrient under test.

Casein hydrolyzate was prepared from alcohol-extracted, twice-reprecipitated casein by sulfuric acid hydrolysis, followed eventually by treatment with norit as mentioned above for glucose (Landy and Dicken, 1942). The purest available preparations of amino acids, vitamins, and other supplements were used; whenever possible, synthetic products were chosen. Inorganic chemicals were cp or equivalent. When storage was necessary, stock solutions were preserved with chloroform-toluene at 2 C.

Every effort was made to minimize the carry-over of growth-affecting substances in the inoculum. Test media were inoculated by means of Pasteur capillary pipettes with a drop of a light suspension in the basal medium of young agar-grown cells. Estimations by the plate count method showed that 10^4 to 10^5 viable cells were transferred to each flask containing 10 ml of the test medium. As a test of the efficiency of this dilution flask technique in eliminating any noticeable carry-over effects (see also section V, below), cultures in various test media were transferred serially four times into fresh flasks of the same medium. Inasmuch as results on the fourth transfer were usually identical with the first, or, if anything, the last transfers yielded better growth due to adaptation to the simple medium, serial transfers were later used only in critical or questionable experiments. The purity and viability of the inoculum were always checked by streaking from the dilution flasks at the time of inoculation.

Unless otherwise stated, all cultures were incubated at 28 C. Turbidity was estimated visually or measured by means of an Evelyn photoelectric colorimeter. The 620 $\text{m}\mu$ filter was used in these turbidity measurements to avoid possible interference by varying concentrations of cell pigment; the yellow *Xanthomonas* pigment transmits completely light of that wave length (Starr, 1944). Turbidity is expressed in terms of "density" or "L-value." These quantities were calculated from the Evelyn galvanometer readings by means of the relationship: $D = \log \frac{G_o}{G_t}$, where D is the "density"; G_o equals the galvanometer reading, ordinarily 100, corresponding to the uninoculated medium; and G_t indicates the galvanometer reading of the culture.

III

The data in table 2 show that many, but by no means all, of the tested *Xanthomonas* cultures grew in the NH_4Cl , glucose, and salts basal medium without supplement. Those cultures which grew in the basal medium ("nonexacting" strains) did so at a rate somewhat lower than in nonsynthetic media; although,

with sufficient buffer, the final crops were about the same. For example, the usual peptone or yeast extract media permitted moderate to heavy growth of

TABLE 2

Ability of Xanthomonas isolates to grow in the NH₄Cl, glucose, and salts basal medium and in the basal medium supplemented by glutamic acid and methionine

SPECIES	NUMBER OF ISOLATES	VIRULENCE*	YEAR ISOLATED OR RECEIVED	GROWTH IN BASAL MEDIUM PLUS			
				Nil	Glutamate plus methionine	Glutamate	Methionine
<i>X. barbareae</i>	2		1939	+++†	++	++	++
<i>X. begoniae</i>	4		1939-1945	++	++	++	++
<i>X. campestris</i>	8	v+	1936-1944	++[2 ++]	++	++	++
var. <i>armoraciae</i>	1		1939	++	++	++	++
<i>X. carotae</i>	1		1939	++	++	++	++
<i>X. corylina</i>	3		1935-1941	++	++	++	++
<i>X. cucurbitae</i>	1		1933	++	++	++	++
<i>X. geranii</i> . .	3		1936-1940	+[1 ++]	++	++	++
<i>X. hederac</i>	3	v+[1 v?]	1932-1944	0	++	0?	++
<i>X. incanae</i> . .	2		1940-1941	++	++	++	++
<i>X. juglandis</i>	9	v+	1940-1945	+[1 ++; 1 0?]	++	++	++
<i>X. lespedezae</i>	2	v+	1944-1945	++	++	++	++
<i>X. maculafolagardeniae</i>	2	v+	1945	+[1 ++]	++	++	++
<i>X. malvacearum</i>	7		1941-1945	+++[1 ++; 1 0]	++	++	++
<i>X. manshotis</i> . .	6		1941	+[2 ++]	++	++	++
<i>X. papavericola</i>	2		1932-1943	+[1 ++]	++	++	++
<i>X. pelargonii</i>	4		1938-1942	+[1 0?]	++	++[1 +]	++[1 +]
<i>X. phaseoli</i>	5	v+	1941-1944	+[1 0?]	++	++[2 +]	++[2 +]
var. <i>fuscans</i>	3	v+	1944	0	++	+	+[1 0?]
var. <i>sojense</i>	6	v+	1930-1944	+	++	++	++
<i>X. pruni</i>	8	v+	1941-1945	0	0?§		
<i>X. tarazaci</i>	1		1942	+	++	++	++
<i>X. translucens</i> f. sp. <i>cere-</i>							
alis	1	v+	1942	0	++	0?	++
f. sp. <i>cerealis</i> (?)	2	v+	1942	0	++	0?	++
f. sp. <i>cerealis</i> or <i>undulosa</i>	2	v+	1941-1942	0	++	0?	++
f. sp. <i>hordei</i>	1	v+	1938	0	++	0?	++
f. sp. <i>hordei-avenae</i>	3	v+	1942-1945	0[1 0?]	++	0?	++
f. sp. <i>undulosa</i>	4	v+	1936-1944	0[1 0?]	++	0?	++
<i>X. vasculorum</i>	1		1933	++	++	++	++
<i>X. vesicatoria</i> (from pep-							
per)	7		1937-1939	+[3 ++; 1 0]	++	++[1 0]	++
(from tomato)	5		1932-1943	+[1 0?]	++	++[1 +]	++
var. <i>raphani</i>	1		1940	++	++	++	++
<i>X. vignicola</i>	3		1942	+	++	++	++

* Virulence determined on "usual" host at the time that nutritive requirements were studied. Legend: v+, distinctly virulent; v?, weakly or questionably virulent; v0, avirulent; no sign, virulence not determined at this time.

† ++ = moderate to heavy growth at a rate comparable to that observed in peptone media under similar conditions of inoculation and incubation; i.e., good growth within 3 to 6 days. + = subcultivable and eventually heavy growth, but at a distinctly lower rate than is indicated by "++". 0? = very slow development, starting only after 2 or 3 weeks' incubation, and possibly representing an adaptation to the medium rather than sufficient synthetic ability. 0 = no growth, even after prolonged incubation.

‡ The number of isolates deviating from the average reaction is shown in brackets together with the type of deviation.

§ An "average" value. Results in replicate experiments ranged from "0" to "+". See discussion in text.

most *Xanthomonas* cultures from small inocula within 2 or 3 days, whereas, in the synthetic medium, comparable development of the "nonexacting" isolates usually took 4 to 10 days or even longer. Moreover, *X. hederac*, *X. phaseoli* var.

fuscans, *X. pruni*, and *X. translucens*, as well as a few equally "fastidious" isolates of other species, failed to grow at all in the basal medium alone.⁶

Addition of 0.5 per cent "vitamin-free" casein hydrolyzate to the basal medium not only stimulated the growth of the "nonexacting" isolates, but also supplied the essential growth factors required for the prompt development of the "fastidious" cultures with the exception of *X. pruni*. Search for the stimulation factors and essential growth factors of casein hydrolyzate was facilitated by the observation that a mixture of amino acids produced identical effects. By systematic omission of amino acids from this mixture, individually and in groups, glutamic acid and methionine were inferentially identified as active ingredients for several typical cultures, since when both were omitted growth was again slow or nil as in the unsupplemented basal medium. This activity was verified by the prompt, luxuriant growth of every culture used in this study (except *X. pruni*, as discussed below) when synthetic *dl*-glutamic acid (0.1 per cent) and *dl*-methionine (0.02 per cent) were added to the basal medium.

The preceding statement is not to be interpreted as meaning that *both* glutamic acid and methionine were "needed" by *all* cultures. Indeed, reference to table 2 reveals that only a scant minority of the cultures (e.g., 3 *X. phaseoli* var. *fuscans*; 2 *X. phaseoli*) depended upon addition of both amino acids for prompt, full development.

Most of the cultures studied developed practically as well with either glutamic acid alone or methionine alone as the sole additional nutrilit. For the majority of this group, the single amino acid simply stimulated growth; however, there are a few examples of obligate requirement which could be satisfied by either of the amino acids. Preliminary experiments indicate that the nutrilit needs of at least some members of this class can also be met by substances other than these two amino acids.

In contrast to the foregoing categories, there are cultures which seem to depend primarily and apparently obligately on methionine; in this division are all available isolates of *X. hederæ* and *X. translucens*⁷ and rare exceptional strains of other species. Prompt growth of these cultures depended absolutely upon the presence of methionine in the medium. Occasionally, scanty development began in methionine-free media after incubating 10 or 20 days, possibly because of adaptation such as described for some species of marine luminous bacteria by Doudoroff (1942). However, most cultures of *X. hederæ* and *X. translucens* did not grow at all unless methionine was present, and, moreover, in its presence there was *prompt* development of *all* isolates of these two species. Figure 2 demonstrates this relationship in the case of a typical isolate and indicates the range of active methionine concentrations. In the presence of sufficient methionine, there was little or no stimulation of growth of these species by

⁶ Unquestionably, the dividing line between the more slowly developing "nonexacting" isolates and the "fastidious" isolates is indistinct. If observations had not been continued beyond 7 or 10 days, a number of the slower growing "nonexacting" cultures would have appeared as "fastidious."

⁷ *X. translucens*, as used herein, includes the *formae speciales* of Hagborg (1942) as well as the pathogenetic forms of Wallin (1944a; 1944b).

glutamic acid. For many isolates of these two species, methionine could be replaced by homocysteine, or by homocysteine plus choline, but a reduced growth rate resulted from these substitutions.

As noted above, *X. pruni* did not grow in the unsupplemented basal medium. Even when "vitamin-free" casein hydrolyzate was added to the basal medium, this species did not grow, or began to develop only after 2 or 3 weeks' incubation, in sharp contrast to all other species of *Xanthomonas*, which grew luxuriantly and quickly with casein hydrolyzate. All available isolates of *X. pruni* grew well when Difco yeast extract was added to the casein hydrolyzate medium, and a mixture of B vitamins could be substituted for the yeast extract with no discernible loss of activity. By successively omitting one or two growth factors at a time from the vitamin mixture, nicotinic acid or nicotinamide was indicated

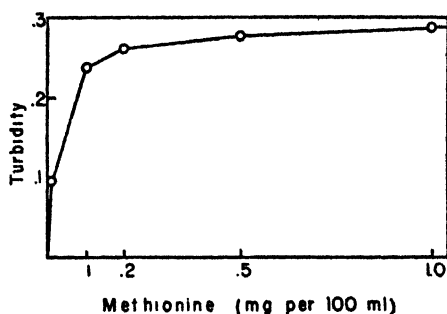


FIG. 2. EFFECT OF CONCENTRATION OF *DL*-METHIONINE, ADDED TO THE BASAL MEDIUM, ON THE GROWTH OF *XANTHOMONAS TRANSLUCENS* ISOLATE XT12

Turbidity measured after 5 days' incubation and expressed in "density" units. The turbidity at 2 to 100 mg of methionine per 100 ml was at the level shown at 1 mg; above 100 mg the growth fell off to practically none at 1,000 mg. These results are typical of those obtained with all available isolates of *X. translucens* and *X. hederae*.

as the active ingredient. All cultures of *X. pruni* grew well in the casein hydrolyzate medium plus nicotinic acid; figure 3 shows the quantitative response to the vitamin. Nicotinamide could replace the free acid.

In the presence of nicotinic acid, casein hydrolyzate could be substituted by glutamic acid and methionine as for other *Xanthomonas* species; somewhat slower growth occurred with either of the amino acids used individually, and there was no growth in the basal medium supplemented only by nicotinic acid.

Under certain conditions, there was growth of some *X. pruni* cultures in the supposedly nicotinic-acid-free glutamate and methionine medium—at times suggestive of a fluctuating contamination by nicotinic acid. After actual contamination from the glassware, medium components, and inoculum had been ruled out, certain cases of this seeming contamination were tentatively interpreted as the formation, during autoclaving of the media in the various experiments, of nicotinic acid or a substitute therefor, varying in concentration as a function of as yet undetermined conditions. This is probably similar to the phenomenon studied by Bovarnick (1943, 1944) in which a substance showing nicotinamide activity for *Shigella dysenteriae* (in one case, actually isolated and

identified chemically as nicotinamide) was formed by prolonged heating of glutamic acid, methionine, and other amino acids. Certain other cases of growth in the nicotinic-acid-free amino acid medium, not amenable to the foregoing interpretation, may represent synthesis of nicotinic acid from the amino acids; although, in this connection, it is puzzling why such a synthesis could proceed in the amino acid medium, but is not nearly so evident in the casein hydrolyzate medium, which, after all, does contain similar quantities of the two amino acids.

There can be no doubt that nicotinic acid was distinctly stimulatory for the growth of all *X. pruni* isolates used in this study; in certain cases the vitamin was indispensable. The requirement of *X. pruni* for nicotinic acid serves to distinguish this organism from the other species of *Xanthomonas*, since none of the latter were affected by nicotinic acid (see figure 3) or, for that matter, by a mixture of 12 water-soluble vitamins. From the determinative standpoint, it

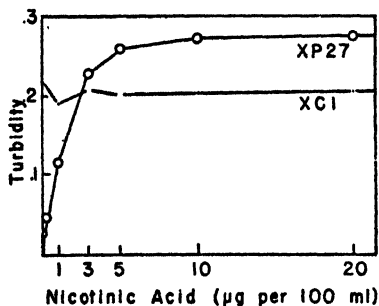


FIG. 3. RESPONSE OF A TYPICAL ISOLATE (XP27) OF *XANTHOMONAS PRUNI* TO NICOTINIC ACID IN A CASEIN HYDROLYZATE MEDIUM

For comparison, there is shown the "response" of an isolate of *X. campestris* (XC1), illustrating the indifference of all species other than *X. pruni* toward the vitamin. Turbidity measured after 3 days' incubation and expressed in "density" units.

would be best to test for the nicotinic acid requirement by using casein hydrolyzate media until such time as the anomalous behavior shown in the nicotinic-acid-free glutamate and methionine medium be clarified.

The inability of *Xanthomonas* species to grow with asparagine as the sole source of both carbon and nitrogen has been reported by Burkholder (1939) and confirmed by Starr and Weiss (1943). Because the majority of xanthomonads are now shown to be able to grow in simple media, it is of interest to examine the reason for the unsuitability of asparagine. By the study of several "non-exacting" isolates, it was learned that the addition of glucose to the asparagine medium resulted in growth at least as good as in the present NH_4Cl glucose basal medium. This might suggest that the incompetence of asparagine is due to its inappropriateness as a carbon or energy source, although it suffices as a nitrogen source.

IV

These findings on the nutrition of *Xanthomonas* may be useful in clarifying the taxonomy of the group. Since it is established that all the species studied

have comparatively simple nutritive requirements, a statement of these general needs might well be appended to the description of the genus. Perhaps, such information would eventually be helpful in establishing the affinities of the genus *Xanthomonas* in the family Pseudomonadaceae or elsewhere.

Minimal nutritive requirements may aid in distinguishing species within the genus; for example, the nicotinic acid requirement of *X. pruni*, and the methionine need of *X. hederæ* and *X. translucens*. In using these properties in determinative bacteriology, one must bear in mind the experimental conditions under which these differences have been demonstrated. Only when small inocula, highly purified media components, and scrupulously cleaned glassware are employed can clear-cut results of the sort reported herein be expected. Furthermore, the well-known phenomenon of mutability of nutritive requirements must always be considered.

In this connection, however, the data in table 2 show relatively few instances in which individual isolates deviate qualitatively from the reaction characteristic of the species; nor is there any apparent trend toward less exacting requirements for the isolates which had been cultivated for the longest period on laboratory media. The latter is interesting in view of frequent references in the literature to the effect "that organisms as directly isolated from cases of disease have more exacting nutrient needs, and that in culture *in vitro* they become trained to grow in simpler media than they require when first isolated" (Knight, 1938, p. 152). In several cases in the present study, cultures just two or three transfers away from the host plant (the bare minimum considering the manipulations involved in isolating and purifying cultures) were shown to have precisely the same nutritive requirements as stock cultures of the same species which had been grown on artificial media for as long as 10 or 15 years. This was true of "nonexacting" species as well as of methionine- and nicotinic-acid-requiring species. The cause of this seeming stability does not lie in an inherent immutability, for it was possible to train some "fastidious" cultures to dispense with an initially "needed" growth factor; probably, it may be attributed to the fact that the stock culture media undoubtedly contained the few simple required nutrilites and, consequently, did not favor any regaining of synthetic ability.

V

It might not be amiss at this point to consider briefly the relationship of the exact nutritive requirements of *Xanthomonas* to the media used for determinative "fermentation" tests. As Elliott (1930) and Burkholder (1932) have emphasized, peptone basal media are unsuited for "fermentation" tests of *Xanthomonas* because of a prominent alkali production from the peptone with a consequent masking of any acid which may have been formed from the carbon source. Unfortunately, results with the suggested substitute, the synthetic carbohydrate medium of the *Manual of Methods for Pure Culture Study of Bacteria* (Comm. Soc. Am. Bact., 1923-1936), may be equally misleading for some species of *Xanthomonas*. Since the recommended synthetic basal medium does not provide the accessory growth factors required by the "fastidious" species, the failure of an organism to grow and produce a pH change in the synthetic medium plus

carbon source might result from lack of an essential growth factor rather than from an inability to dissimilate the carbon source.

Another source of confusion is the variable carry-over of growth factors in the inoculum, especially when transfers are made directly from stock cultures. Although this would have little effect on carbon source experiments made in peptone media, it is highly important in synthetic media, particularly with "fastidious" species. This fact is brought out by scores of observations in the present study which show that when the media were seeded heavily with a loopful of cells from stock cultures many of the "fastidious" *Xanthomonas* cultures grew in the unsupplemented basal medium. (This sort of experimentation quite likely led to the stated belief that *all* species of *Xanthomonas* grow in ammonium glucose media.) With much smaller inocula and thereby reduced carry-over of growth factors, such as is provided by inoculation via a dilution flask, there is no growth of these "fastidious" species. Between these extremes of inoculum size, growth would or would not occur depending upon the quality and quantity of growth factor carried over.

That the foregoing is not a purely rhetorical discussion is readily apparent from the discrepant results shown in the literature. One must guard against the lack of an essential growth factor in the basal medium, either by the somewhat uncertain method of using very large inocula, or preferably, by fortifying the basal medium with the proper accessory growth factor(s). If exact information on this subject be lacking, a small quantity of yeast extract (e.g., 0.02 to 0.05 per cent of Difco yeast extract) can usually be depended upon to supply the missing nutrients without interfering with the desired pH changes.

VI

From the preceding it is evident that the nutritive requirements of all tested *Xanthomonas* species could undoubtedly be met by the tissues of practically any plant. Why, then, are these phytopathogenic bacteria restricted to specific, or to a limited series of, host plants? The answer clearly does not lie in simple satisfaction of the minimal nutritive requirements of the pathogen. This is in agreement with Chester's (1933) analysis of the analogous mycological situation. Perhaps an explanation can be found in chemical inhibitors such as plant acids or phenols (Link and Walker, 1933). In view of recent reports concerning the occurrence of antibiotic substances in higher plants (Pederson and Fisher, 1944; Lucas and Lewis, 1944; Huddleson *et al.*, 1945), it is tempting to speculate on the existence in plants of *specific* antibiotics which are capable of inactivating phytopathogens other than the particular bacterial species which are able to infect that host.

SUMMARY

The minimal nutritive requirements of 113 isolates of phytopathogenic bacteria belonging to 30 species and varieties of the genus *Xanthomonas* were determined under conditions of controlled inoculum, carefully cleaned glassware, and pure medium components.

Under these experimental conditions, it was learned that most, but not all,

species of *Xanthomonas* can grow to some extent in the simplest medium used: NH_4Cl , glucose, and salts. Methionine, glutamic acid, and nicotinic acid in various combinations served to furnish the stimulation factors and essential growth factors necessary for the prompt development of the more exacting species.

Growth in these simple media shows promise of utility as a taxonomic character on the generic and specific levels.

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THE IN VITRO ACTION OF SULFONAMIDES AND PENICILLIN ON ACTINOMYCES

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Since the comparative effectiveness of sulfonamides and penicillin on actinomycetes is of therapeutic interest, the action of these chemotherapeutic agents on two strains of *Actinomyces* was studied *in vitro*.

Strain A (case 1) was isolated from a case of actinomycosis of the soft tissues over the right mandible. The patient received 40,000 units of penicillin intramuscularly every 3 hours for 60 doses, a total of 2,400,000 units. The case was considered a good therapeutic result and is being reported by Major Victor N. Tompkins.

Strain B (case 2) was isolated from a case of actinomycosis of the right pleural cavity. Following surgical drainage, 40,000 units of penicillin were given intramuscularly every 3 hours for a total of 5,000,000 units. There was clinical improvement, but the sinus tracts failed to close and a slight purulent discharge persisted. One month after the termination of penicillin therapy, cultures of the purulent material contained *Actinomyces*. Further surgical drainage was performed and 2,400,000 units of penicillin were administered intramuscularly. In addition, the empyema cavity was irrigated with a solution of penicillin, 5,000 units per ml. Ten days after the cessation of therapy, the *Actinomyces* was still present in purulent material discharged from the sinus tracts. At that time the patient was evacuated from the hospital.

Both strains, A and B, were isolated from granules washed out of purulent material draining from the sinus tracts. The morphological and cultural characteristics of these strains are summarized in table 1.

METHODS

The *in vitro* action of sulfadiazine, sulfathiazole, and penicillin on these two strains of *Actinomyces* was studied in the following manner. One-tenth ml of a 96-hour broth culture of each strain was inoculated into tubes of glucose (1.0 per cent) nutrient agar containing varying concentrations of a sulfonamide or of penicillin as indicated in table 2. This amount of inoculum contained a few scarcely visible particles. The inoculum was thoroughly mixed with the agar and the culture was incubated at 37 C. Density readings were taken after 96 hours. Growth in the control culture, without any drug, was evaluated as 4+, and the growth in the other tubes was comparatively estimated. No further relative change was observed in the cultures after 30 days.

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Morphological and cultural characteristics of strains of Actinomyces

	Strain A	Strain B
Granule	Minute, smooth, grayish white, easily crushed	0.1-0.4 mm in diameter, yellowish white, hard irregular spheres, resistant to crushing
Morphology*	Tangled branching filaments with some granular staining; in culture, terminally clubbed bacillary forms and few degenerated very thin gram-negative filaments	Tangled branching filaments with some granular staining; in culture, few terminally clubbed bacillary forms and few degenerated very thin gram-negative filaments
Motility	Nonmotile	Nonmotile
Gram stain	Positive	Positive
Acid-fast 1% H ₂ SO ₄ for 5 minutes	Partly decolorized	Completely decolorized
Oxygen requirement	Microaerophilic	Anaerobic
Temperature requirement	Growth at 37 C; no growth at 22-25 C	Growth at 37 C; no growth at 22-25 C
Growth in 1.0% glucose agar	Profuse, discrete white colonies after 72 hours; no growth within 0.5 cm of surface	Discrete white colonies after 96 hours; colonies resistant to crushing; no growth within 0.5 cm of surface
Growth in brain heart infusion broth	Slow granular growth in bottom of tube	No growth
Growth in fluid anaerobic thio-glycollate medium	Profuse, granular	Profuse, granular

* See figures 1 and 2.

TABLE 2

In vitro action of sulfathiazole, sulfadiazine, and penicillin on two strains of Actinomyces

DRUG	CONCENTRATION	GROWTH IN 96 HOURS AT 37 C	
		STRAIN A	STRAIN B
Sulfathiazole	10 mg per cent	+	
	5 " " "	++	
	2 " " "	+++	
	1 " " "	+++	
Sulfadiazine	20 " " "		+
	10 " " "	+	+++
	5 " " "	++	
	2 " " "	+++	
	1 " " "	+++	++++
Penicillin	1.0 unit per ml	none	none
	0.5 " " "	none	none
	0.2 " " "	none	none
	0.1 " " "		none
	0.1 " " "		++
	0.001 " " "		+++
No drug, control tube		++++	++++

No further changes noted in cultures after 30 days.

RESULTS

The results are summarized in table 2.

The growth-inhibiting actions of sulfadiazine and sulfathiazole on strain A are approximately equal, but concentrations of 10 mg per cent failed to prevent growth.

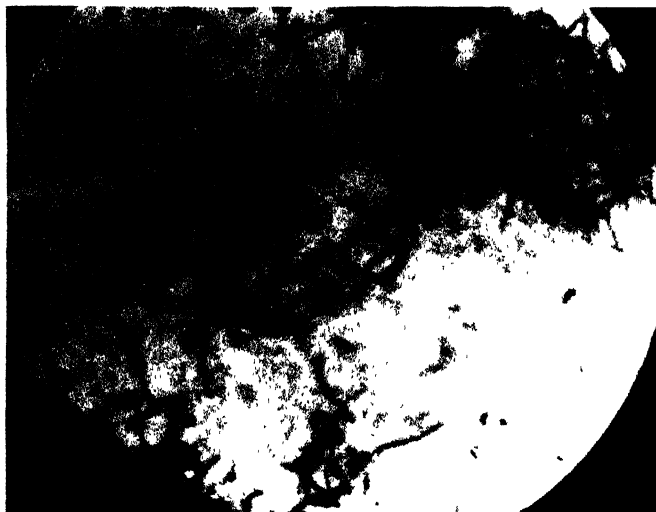


FIG. 1. PHOTOMICROGRAPH OF STRAIN B, 96 HOUR CULTURE IN THIOLYCOLATE BROTH (GRAM STAIN, MAGNIFICATION, $\times 970$)

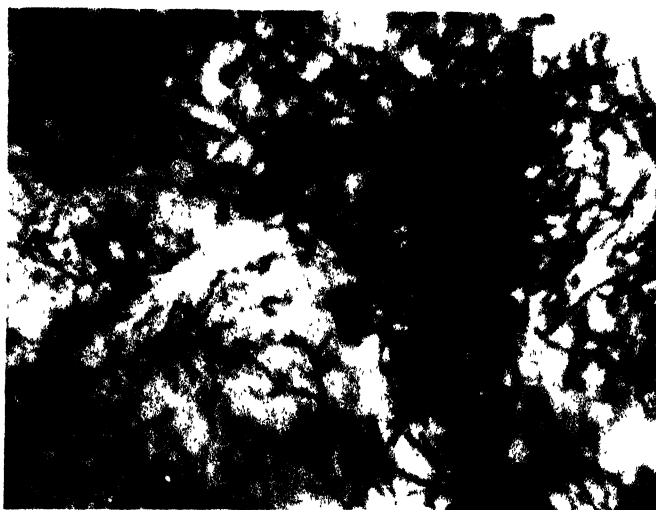


FIG. 2. PHOTOMICROGRAPH OF STRAIN A, FILAMENTS IN A CRUSHED GRANULE OBTAINED FROM PURULENT MATERIAL (GRAM STAIN; MAGNIFICATION, $\times 1,290$)

The growth of strain B was not inhibited so much by sulfadiazine as was strain A, and even 20 mg per cent of sulfadiazine failed to prevent completely the growth of strain B.

Two-tenths units of penicillin per ml of medium prevented the growth of both strains of *Actinomyces in vitro*. The growth of strain B was prevented by 0.1 unit per ml, and 0.01 unit per ml resulted in a demonstrable inhibition of growth of this strain.

DISCUSSION

These two strains of parasitic *Actinomyces* showed individual morphological and cultural differences, but both may be classified under the provisional heterogeneous species, *Actinomyces israeli*, as suggested by Rosebury (1944).

Although sulfathiazole and sulfadiazine, in the higher concentrations employed *in vivo*, had some growth-inhibitory action *in vitro* on these two strains, it was not sufficient to warrant sulfonamide therapy in either case. On the other hand, the amount of penicillin necessary to prevent growth of the two strains *in vitro*, 0.1 to 0.2 units per ml of medium, was within the range of the levels attained in the blood, ascitic fluid, and edema fluid of man (Struble and Bellows, 1944; Cooke and Goldring, 1945; Zinnaman and Seeberg, 1945). Whether or not such concentrations are attained within inflammatory tissue or in empyema cavities could not be ascertained from the available literature.

SUMMARY

The ability of sulfathiazole, sulfadiazine, and penicillin to inhibit the *in vitro* growth of two strains of *Actinomyces* was studied. The sulfonamides, in 10 to 20 mg per cent concentrations, exerted some growth-inhibiting effect. Penicillin prevented the growth of both strains in concentrations of 0.1 to 0.2 units per ml of medium.

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ELECTROKINETIC STUDIES ON BACTERIAL SURFACES

I. THE EFFECTS OF SURFACE-ACTIVE AGENTS ON THE ELECTROPHORETIC MOBILITIES OF BACTERIA

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The microscopic method of electrophoresis has frequently been used in investigations of the electrokinetic behavior of bacteria. Abramson, Moyer, and Gorin (1942) have pointed out the advantages of the method and consider it to be one of the most powerful tools for the investigation of surface phenomena of living cells. The early literature on the application of the methods of electrophoresis has been reviewed by Abramson (1934). In this review the electrophoretic behavior of bacterial cells was discussed particularly in relation to their surface isoelectric points, immune reactions, virulence, suspension stability, and surface composition.

Moyer (1936b) reported that the surface factors which determine the electrophoretic mobility in mature cultures of *Escherichia coli* were constant during long periods of cultivation, provided rough variants were eliminated. On the other hand, young cultures exhibited a lowering of electrophoretic mobility during the time corresponding to the "period of physiological youth." More recently, Frampton and Hildebrand (1944) have reported that *Erwinia amylovora* and *Phytomonas stewartii* show distinctive mobility-pH curves though with neither organism was there a correlation of electrophoretic mobility with virulence.

The method of electrophoresis has been used to obtain evidence of surface changes associated with bacterial dissociation. Joffe and Mudd (1935) found that four strains of smooth, nonflagellate intestinal bacteria have approximately zero mobility over wide ranges of pH and salt concentration. Moyer (1936b) confirmed this behavior with *Salmonella gallinarum*. He further found that smooth strains of *Escherichia coli* maintain an almost constant electrophoretic mobility over a wide pH range, whereas the mobility of rough strains varies with pH. Stearns and Roepke (1941a, 1941b) noted a similar behavior with smooth and rough variants of *Brucella abortus* and were able to follow the course of the dissociation process in a quantitative way by measuring the electrophoretic mobilities.

Moyer (1936b) called attention to the fact that the net charge density-concentration of electrolyte curves of both smooth and rough variants of *Escherichia coli* are similar to those of some carbohydrate and lipid surfaces. The effect of varying pH and electrolyte concentration on the electrophoretic mobility of organic and inorganic surfaces of various types has been reviewed by Abramson (1934) and Abramson, Moyer, and Gorin (1942). It is clear that the response

of many surfaces to varying pH and electrolyte concentration is not always sufficiently characteristic to warrant the use of the electrophoretic method for purposes of identification. With regard to bacteria, electrophoretic studies have not been particularly fruitful in supplying information as to the nature of the surface cellular constituents which determine the electrokinetic potential.

The cytological and immunological evidence on the outer structures of bacterial cells has been discussed by Knaysi (1944) and Dubos (1945).

It is generally considered that the protoplasm of a bacterial cell is surrounded by a cytoplasmic membrane which is, in turn, enclosed by a cell wall. The cell wall may be surrounded by a slime layer or capsule. There is relatively little information as to the chemical composition of the cell wall of bacteria, though claims have been made that it consists of cellulose, hemicellulose, or chitin. There is lack of agreement as to the origin and character of the slime layer, though the capsular substances of a number of bacterial species consist essentially or exclusively of high molecular weight polysaccharides (Dubos, 1945). Knaysi (1944) has stated, "The chemical composition of the slime layer, like that of the cell wall, has been studied both microchemically and macrochemically and, as in the case of the cell wall, we are only certain that it may be different in different bacteria and that it contains carbohydrate."

It seemed reasonable to expect that the electrokinetic behavior of bacteria upon treatment with suitable reagents and under carefully controlled conditions would be primarily dependent upon the nature of the surface cellular constituents. If this be true, it should be possible more precisely to identify the substances present in bacterial surfaces by comparison with surfaces of known composition. In seeking such a method we treated bacterial cells with surface-active agents with the expectation that they might produce well-defined changes in mobility dependent on the chemical nature of the materials predominating in the surface.

The mobilities of protein and lipid and other substances were expected to be distinctively altered by treatment with surface-active agents because of their different affinities for the hydrocarbon or polar groups of the wetting agent molecules. For example, a negatively charged protein surface might have some of its negative charges neutralized by the polar groups of a cationic surface-active agent, whereas it would probably not be affected by an anionic surface-active agent. If a surface were lipid in which the hydrocarbon end of the wetting agent molecule were soluble, the cationic wetting agent should again decrease the negative charge, but at different concentrations than with a protein; and an anionic wetting agent should increase the charge. Powney and Wood (1940) demonstrated such a behavior with mineral oil droplets treated with cationic and anionic surface-active agents.

As an initial step it was necessary to determine whether the electrokinetic behavior of various bacteria on treatment with surface-active agents would be consistent and characteristic. With this in view, a number of bacteria from widely different taxonomic groups have been treated with surface-active agents and the resulting electrophoretic mobilities measured.

METHODS

Electrophoresis apparatus. Electrophoresis measurements were made in two Briggs' microelectrophoresis cells (Briggs, 1940), employing the standard method for the investigation of electrophoresis described by Moyer (1936a).

Bacteria. To minimize possible variations in surface due to environment, the species studied were grown under as nearly comparable conditions as practicable. The cultural conditions employed are shown in table 1. In a few cases it was desirable to investigate the influence of environment on the surface, so other media were used as well.

TABLE 1
Culture conditions for the bacteria investigated

BACTERIA	MEDIA	TIME OF GROWTH	METHOD OF AERATION	TEMPERATURE, C
<i>Micrococcus aureus</i>	F.D.A. broth*	20-28 hours	Shaking machine	34-37
<i>Escherichia coli</i>	F D.A. broth	20-28 hours	Shaking machine	34-37
<i>Pseudomonas aeruginosa</i>	F.D.A. broth	20-28 hours	Shaking machine	34-37
<i>Spirillum volutans</i>	Nutrient broth	20-28 hours	Shaking machine	Room temperature
<i>Bacillus pseudotetanicus</i>	Nutrient agar	20-28 hours		34-37
<i>Mycobacterium smegmatis</i> and <i>phlei</i>	Glycerol broth	4-6 days		34-37
<i>Chondrococcus columnaris</i>	0.5% tryptone broth	48 hours	Shaking machine	Room temperature
<i>Chondrococcus columnaris</i> cysts	0.5% tryptone broth	7 days		Room temperature
<i>Spirochaeta</i> sp.	10% red blood cell agar	5-8 days		Room temperature

* The medium employed in the Food and Drug Administration method of testing disinfectants.

The bacteria were harvested and washed three times in phosphate buffer of ionic strength 0.02 and at pH 6.9. The bacterial cells were added to the buffered solutions of surface-active agents from stock suspensions containing 5 per cent cells by volume. It was determined that the number of bacteria, within limits reasonable for observation, had no effect on the charge acquired in a given concentration of wetting agent. It was also determined that the time the readings were made after suspending the cells in solutions of surface-active agents had no influence on the charge acquired. However, the suspensions were allowed to stand 5 minutes or more before readings were begun.

Reagents. All experiments were performed in buffer solutions of 0.02 ionic strength. Ordinarily M/100 phosphate buffer at pH 6.9 was used. For more acid solution, acetate buffers (Cohn, 1928) were used. The surface-active agents used were sodium tetradecyl sulfate (2-methyl-7-ethyl undecanol-4-sulfate),¹ an

¹ A specially purified sample, obtained from Dr. F. C. Schmelkes, Wallace and Tiernan Products, Inc., Belleville, New Jersey.

anionic surface-active agent, and cetyl pyridinium chloride,² a cationic surface-active agent. These surface-active agents possessed a satisfactory balance between hydrocarbon and polar groups. The concentrations of surface-active agents employed had no appreciable effect on the ionic strength of the solutions with the exception of those at a concentration of 1×10^{-3} M. Even this concentration increased the ionic strength of the buffers only from 0.020 to 0.021.

Data. With most of the bacteria investigated the values of the mobility at each different concentration were relatively consistent. Usually about ten readings or more were made at each stationary level and averaged. All of the data reported were confirmed by at least two independent experiments. In all experiments, the data were calculated as electrophoretic mobilities expressed in microns per second per volt per centimeter. Graphs have been drawn in which electrophoretic mobility is plotted against concentration of surface-active agent. The concentration is plotted logarithmically; this emphasizes the significant changes in mobility occurring in the dilute concentrations of surface-active agents but minimizes other changes.

EXPERIMENTAL RESULTS

The bacteria investigated were chosen as representatives of widely different taxonomic groups. Included were *Micrococcus aureus* in the family Micrococcaceae, *Escherichia coli* in the family Enterobacteriaceae, *Pseudomonas aeruginosa* and *Spirillum volutans* in the family Pseudomonadaceae, and *Bacillus pseudotetanicus* in the family Bacillaceae, all in the suborder Eubacteriineae. Also included were *Mycobacterium smegmatis* and *Mycobacterium phlei* in the order Actinomycetales, *Chondrococcus columnaris* in the order Myxobacteriales, and *Spirochaeta* sp. in the order Spirochaetales.

Micrococcus aureus

Micrococcus aureus (Insecticide Board strain) was selected as a representative of the gram-positive cocci. The values for the electrophoretic mobilities of *Micrococcus aureus* after treatment with the anionic and cationic surface-active agents are plotted in figure 1. There are several striking points to be noted in the mobility-concentration curves. With the anionic surface-active agent, STS, the mobility increases very slightly, but only in the most concentrated solutions. With the cationic surface-active agent, CPC, not only does the mobility decrease, but the charge is reversed. The decrease in mobility becomes rapid after a concentration of 3×10^{-5} M. The charge is reversed at about 1×10^{-4} M and apparently becomes stabilized after 3×10^{-4} M. No appreciable change in the mobility of *Micrococcus aureus* with pH was observed between pH 4.0 and pH 6.9 when the cells were suspended in buffers of 0.02 ionic strength.

Larson (1922) showed that by growing *Micrococcus aureus* in 3 per cent glycerol broth it was possible to increase the ether-acetone extracts of the cells from 8 to 40 per cent of the dry weight. Such an increase in fatty cell material might

² Purchased from Paragon Testing Laboratories, Orange, New Jersey.

possibly be reflected in the cell surface. To test this possibility a culture of *Micrococcus aureus* was "fattened" by being grown in 3 per cent glycerol broth with aeration. The culture had been previously transferred several times in the same medium.

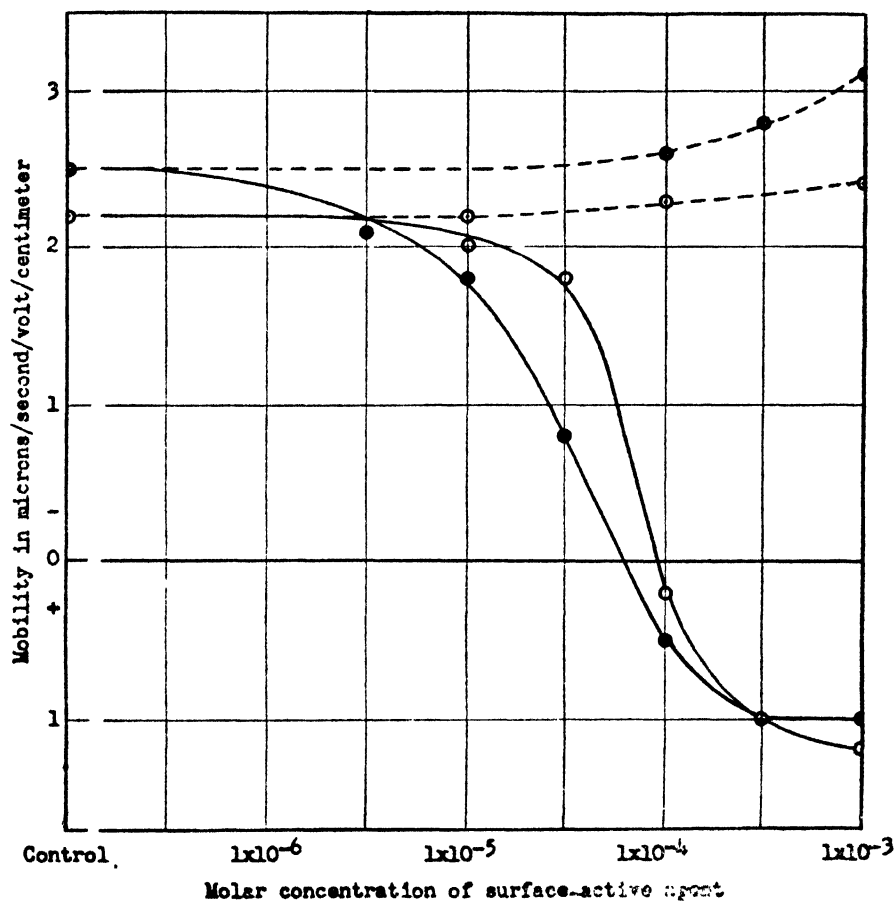


FIG. 1. MOBILITY-CONCENTRATION CURVES FOR *MICROCoccus aureus*

○ — — ○ with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate; "fattened" *Micrococcus aureus*, ● — — ● with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate.

The mobilities of the "fattened" *Micrococcus aureus* are also shown in figure 1. Several differences are apparent in the curves. The initial charge on the fattened cells is somewhat higher, increasing from 2.2 to 2.5 microns per second per volt per centimeter. In this connection, it should be noted that Verwey and Frobisher (1940) reported the electrophoretic velocities of 84 strains of *Micrococcus* to vary from 2.2 to 6.6 microns per second per volt per centimeter in M/75 phosphate buffer at pH 7.4. In the presence of the anionic surface-active agent the fattened cells take on a relatively greater negative charge. With the cationic surface-

active agent the mobility begins to decrease at a more dilute concentration and decreases more gradually, and the charge is reversed sooner. Again the mobility is stabilized in the highest concentrations of CPC.

The increased sensitivity to cetyl pyridinium chloride and the greater negative charge taken on in the presence of sodium tetradecyl sulfate can most logically be explained by an increase in the amount of lipid material in the surface of the cells.

It might be expected that an increase in lipid on the surface of the cells would influence the susceptibility to the action of disinfectants. The germicidal effect of CPC on the normal and fattened strains of *Micrococcus aureus* was determined under precisely the same conditions as those employed in the electrophoretic experiments. At 5- and 15-minute intervals, loopfuls of the suspensions were removed to tubes of melted, cooled agar and plated. As seen in table 2, the

TABLE 2

Resistance of *Micrococcus aureus* and "fattened" *Micrococcus aureus* to cetyl pyridinium chloride

MOLAR CONCENTRATION OF CPC	CONTROL	3×10^{-6}	1×10^{-5}	2×10^{-5}	3×10^{-5}	5×10^{-5}
<i>Micrococcus aureus</i>						
Viable cells after 5 minutes' exposure	>10,000	>10,000	800	200	0	0
Viable cells after 15 minutes' exposure	>10,000	>10,000	200	0	0	0
"Fattened" <i>Micrococcus aureus</i>						
Viable cells after 5 minutes' exposure	>10,000	>10,000	4000	100	0	0
Viable cells after 15 minutes' exposure	>10,000	>10,000	100	0	0	0

resistance of the two cultures to the germicidal action of CPC was essentially the same in spite of the variations in shape of the electrophoretic curves with CPC. Possibly germicidal experiments with disinfectants of other types might show a difference in behavior and provide a clue to the distribution of the increased amount of lipid.

It is interesting to note that a 1×10^{-5} M solution of CPC is toxic even though it has produced a relatively small change in electrophoretic mobility.

Escherichia coli

The strain of *Escherichia coli* (var. *communis*) first selected was used by Ordal and Borg (1942) and Borg (1942) in an investigation of the effects of anionic surface-active agents on certain enzyme systems of *Escherichia coli* and *Micrococcus aureus*. The enzymes of intact cells of *Escherichia coli* were relatively resistant to the inhibitory action of the anionic surface-active agents, but enzyme

preparations made by grinding up the cells showed the same degree of sensitivity as did the enzymes in the intact cells of *Micrococcus aureus*. This suggested that the surface membrane of *Escherichia coli* prevents the penetration of the anionic wetting agents.

It was, therefore, of particular interest to compare the electrophoretic behavior of this strain of *Escherichia coli* with that of *Micrococcus aureus*. The mobility-concentration curves, shown in figure 2, are unlike those obtained with *Micrococcus aureus*. No change in mobility occurs after treatment with the anionic

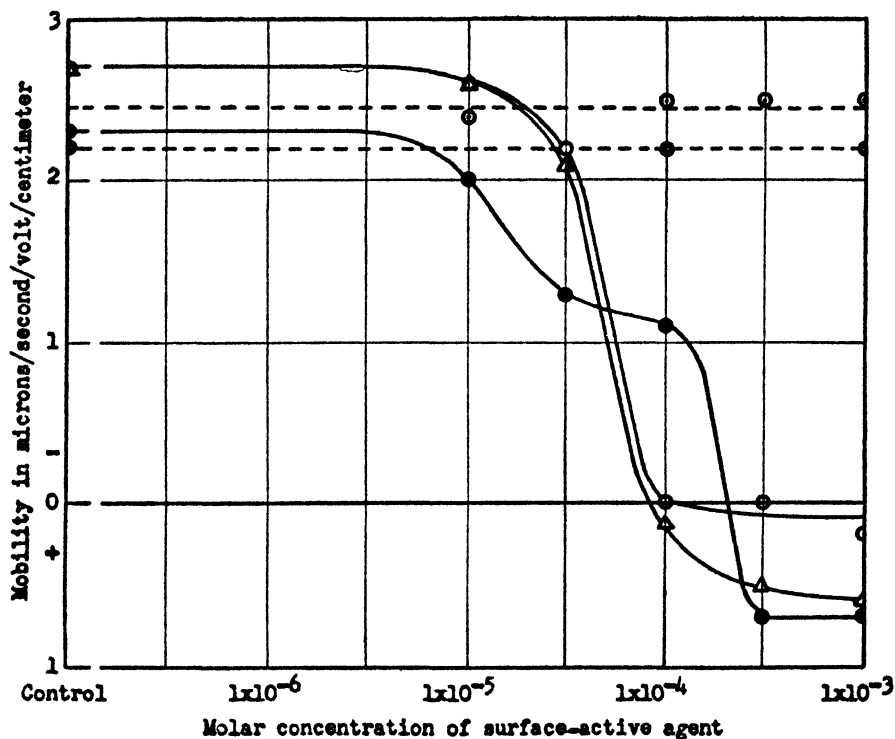


FIG. 2. MOBILITY-CONCENTRATION CURVES FOR *ESCHERICHIA COLI*

Var. *communis*, ●—● with cetyl pyridinium chloride, ○—○ with sodium tetradecyl sulfate; var. *communior*, △—△ with cetyl pyridinium chloride; same strain 7 months later, ○—○ with cetyl pyridinium chloride, ○—○ with sodium tetradecyl sulfate.

surface-active agent, STS. After treatment with the cationic surface-active agent, CPC, the mobility decreases at an irregular rate, which is manifested in the mobility-concentration curve as a hump. The charge is reversed at 2×10^{-4} M, acquires a positive value, and becomes stabilized at 3×10^{-4} M. This culture was smooth, as judged by colony characteristics, turbid growth in broth, and constant electrophoretic mobility with change in pH.

To determine whether the variable rate of change of mobility after treatment with CPC be characteristic of other strains of *Escherichia coli*, a new culture of *Escherichia coli* (var. *communior*) was isolated and tested. The results are

shown in figure 2. The irregular rate of change of mobility is not evident with the new culture. The mobility begins decreasing at about the same concentration as with the first culture, does not show the "hump," and is reversed with a more dilute concentration of CPC. A similar stabilization of charge is evident.

Larson (1922) reported that it was possible to double the ether-acetone extractive material in cells of *Escherichia coli* by growing them in glycerol broth, the procedure used with *Micrococcus aureus*. In order to determine whether this increase in lipid might be reflected in the cell surface, as was the case with *Micrococcus aureus*, suspensions of cells grown in plain broth and in glycerol broth were compared. Identical mobility-concentration curves were obtained. However, the curve, as shown in figure 2, differs from previous curves in one definite respect. The cells acquire almost no positive charge even in the most concentrated CPC solution. This result was confirmed with two more suspensions, one grown in plain broth and one in FDA broth. It should be noted that the last experiments with *Escherichia coli* (var. *communior*) were performed

TABLE 3

Killing and change in mobility of Escherichia coli (var. *communis*) with cetyl pyridinium chloride

MOLAR CONCENTRATION OF CPC	CONTROL	3.12×10^{-6}	6.25×10^{-6}	1.25×10^{-5}	2.5×10^{-5}	5.0×10^{-5}
Mobility and charge	2.20(-)	2.20(-)	1.90(-)	1.86(-)	1.59(-)	1.28(-)
Viable cells after 10 minutes' exposure	113×10^6	106×10^6	76×10^6	19×10^6	0	0

about 7 months after the first experiment. During this interval the stock culture had been occasionally subcultured and stored at icebox temperature. Judged by colony shape and the turbid growth in broth, both cultures were smooth.

Escherichia coli, although resistant to anionic surface-active agents, is readily killed by cationic surface-active agents. It was of interest to determine whether there was any relation between the electrophoretic mobility and the lethal effect in the presence of CPC. Quantitative plate counts were made on suspensions of *Escherichia coli* exposed to varying concentrations of CPC for 10 minutes. Electrophoretic mobilities were determined on equivalent suspensions. The data are recorded in table 3. It is evident that a concentration of CPC that has barely decreased the mobility is lethal in 10 minutes. This behavior is very similar to that observed with *Micrococcus aureus*.

Pseudomonas aeruginosa

Because the several strains of *Escherichia coli* had exhibited such dissimilarities after treatment with wetting agents, *Pseudomonas aeruginosa* was investigated to determine if this behavior might be found in another gram-negative bacterium. Two strains were investigated, the first a laboratory strain which has been subcultured for a period of about 10 years, the second a strain freshly isolated from a kerosene enrichment culture.

The electrophoretic curves of the two, shown in figure 3, are probably the same within the range of experimental error. In the CPC solutions both show the same slow change of mobility beginning at about 3×10^{-5} M. The charge is not reversed until almost 3×10^{-4} M and is stabilized at 3×10^{-3} M. In the STS solutions the mobility shows a small but definite increase.

The consistency of behavior of the two strains of *Pseudomonas aeruginosa* is striking, particularly in view of the variability in behavior of supposedly smooth strains of *Escherichia coli*. Perhaps the most striking characteristic found in

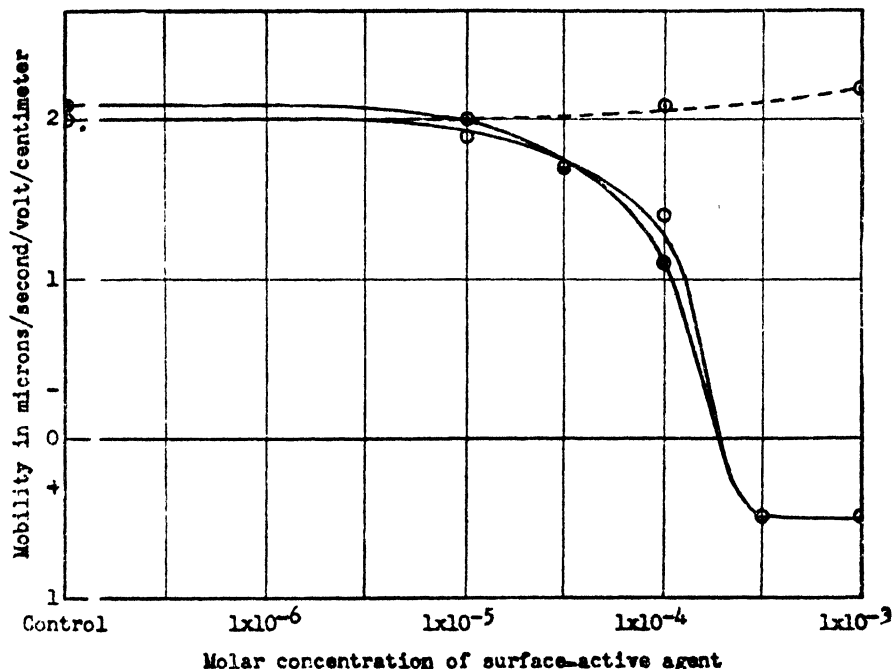


FIG. 3. MOBILITY-CONCENTRATION CURVES FOR *PSEUDOMONAS AERUGINOSA*. Stock culture, ●——● with cetyl pyridinium chloride; new isolate, ○——○ with cetyl pyridinium chloride, ○— —○ with sodium tetradecyl sulfate.

the concentration curves with *Pseudomonas aeruginosa* is the relatively large concentration of CPC required to reduce the charge to zero.

Spirillum volutans

The electrophoretic mobilities of a *Spirillum* closely resembling *Spirillum volutans* after treatment with surface-active agents are shown in figure 4. In the buffer control at pH 7 and in the STS solutions, the values were difficult to obtain because the cells were so extremely motile. The motility ceased completely in more acid solutions and in CPC solutions with concentrations as low as 3×10^{-6} M. If the cells were allowed to stand about an hour in solutions made with freshly boiled and cooled water, enough cells became immotile to permit measurement of the electrophoretic mobility. With the anionic wetting agent the mobility definitely increases. With the cationic wetting agent the mobility begins

to decrease at 1×10^{-5} M, drops rapidly, is reversed at 3×10^{-5} M, and gradually becomes stabilized.

This is the first of the species studied to show a marked sensitivity to pH. At pH 4 the mobility was almost zero. The STS and CPC curves were repeated

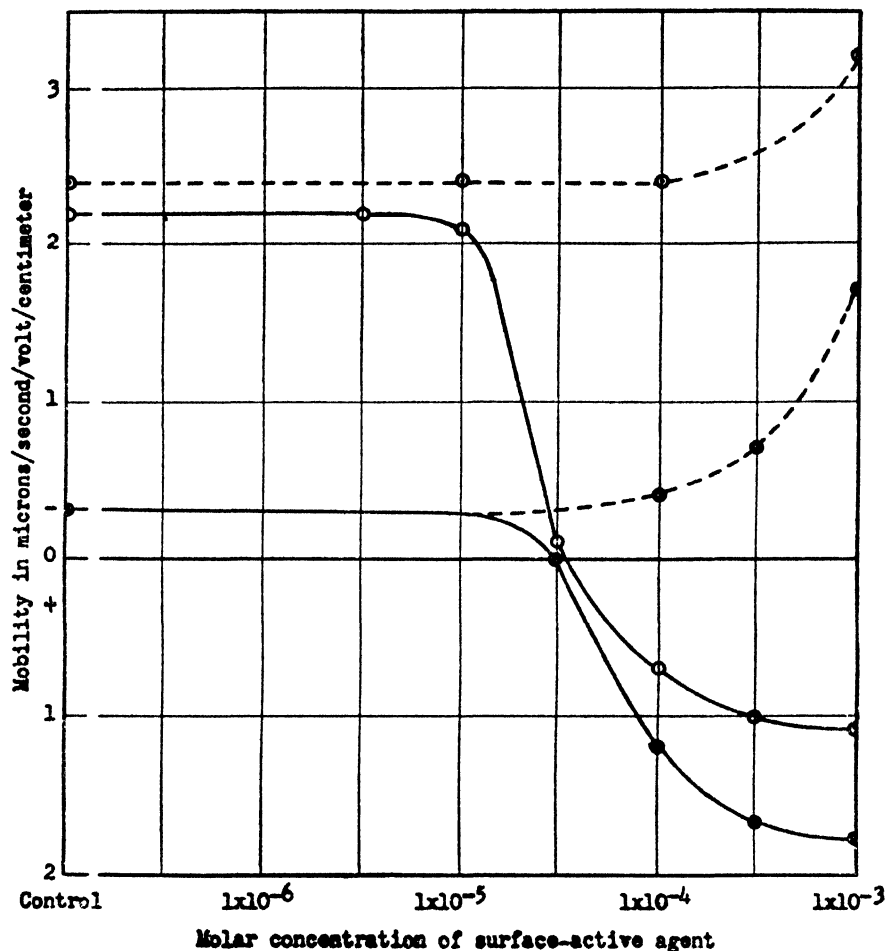


FIG. 4. MOBILITY-CONCENTRATION CURVES FOR *SPIRILLUM VULUTANS*

○ — ○ with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate; at pH 4, ● — ● with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate.

at pH 4. The shape of the curves is similar to those at pH 7, but some of the changes are more pronounced. The mobility increases more with the anionic wetting agent. The positive charge taken on with the cationic wetting agent is greater, but the over-all change in mobility is less.

This strain of *Spirillum* dissociates readily. The usual colonies are smooth and entire, consisting of typical spiral rods. The dissociated colonies are very rough and serrate and made up of nearly straight rods. The two morphological

forms resemble each other in respect to size, flagellation, and the presence of prominent volutin granules. No difference was found in the initial mobilities of the rough and smooth forms. No complete curves have been run on an entirely rough culture, but straight rods occurring in a culture predominantly smooth had the same mobilities as the spiral rods.

Both the spiral and the straight rods displayed polarity. Many of the cells were aligned with the electric field and flipped around when the current was reversed. The polarity is apparently not caused by the flagella, because cells observed in dark field to be flagellated at both ends react the same way.

Bacillus pseudotetanicus

The electrophoretic behavior of both the vegetative cells and the spores of a *Bacillus* (labeled *Bacillus pseudotetanicus* in the University of Washington stock culture collection) was investigated with the results shown in figure 5.

The mobility of the vegetative cells does not change with the anionic surface-active agent. With the cationic surface-active agent, it begins to decrease at 3×10^{-6} M, drops very rapidly, is reversed at 1×10^{-4} M, acquires a large positive value, and is stabilized at 3×10^{-4} M.

The STS curves for the vegetative cells and the spores are similar. The CPC curves, however, are very different. The mobility of the spores begins to decrease much sooner, decreases more gradually, and attains a very different final value.

The mobilities of spores from 4- to 6-day cultures were highly variable, the figures at one extreme in each case closely approximating the values for the vegetative cells. For example, at 1×10^{-3} M CPC, the mobilities of the spores ranged from a slightly negative value to a positive value of 1.3 microns per second per volt per centimeter, whereas the value for the vegetative cells at this concentration was +1.7. This suggested that some of the spores in such a young culture might still retain a part of the vegetative structure at their surfaces. Therefore, a suspension of spores was prepared from a culture 3 months old, and tested. The mobilities, which are those shown in figure 5, were still variable but very much less so. In the most concentrated CPC solutions the values fell into two groups, one slightly negative and the other slightly positive, with an average of about zero mobility.

Probably spores entirely devoid of the vegetative structure would all retain a negative mobility in the most concentrated cationic solutions. This is a result entirely unlike those of all other cells that we have investigated. The surface character of spores is apparently markedly different from the surfaces of both their own and other vegetative cells. This observation may be of some significance in connection with the notable resistance of bacterial spores to adverse conditions.

Mycobacterium smegmatis and *Mycobacterium phlei*

To account for the resistance of bacteria in the genus *Mycobacterium* to ordinary chemical disinfectants, it has sometimes been assumed that a waxy capsule surrounds these cells with resulting impermeability to chemical agents.

Mudd and Mudd (1924, 1927) have presented evidence that acid-fast bacteria have a hydrophobic surface because of their behavior at an oil-water interface.

This observation agrees with the common experience that cells of *Mycobacterium* are wet with difficulty. However, Marton (1943) believes that electron

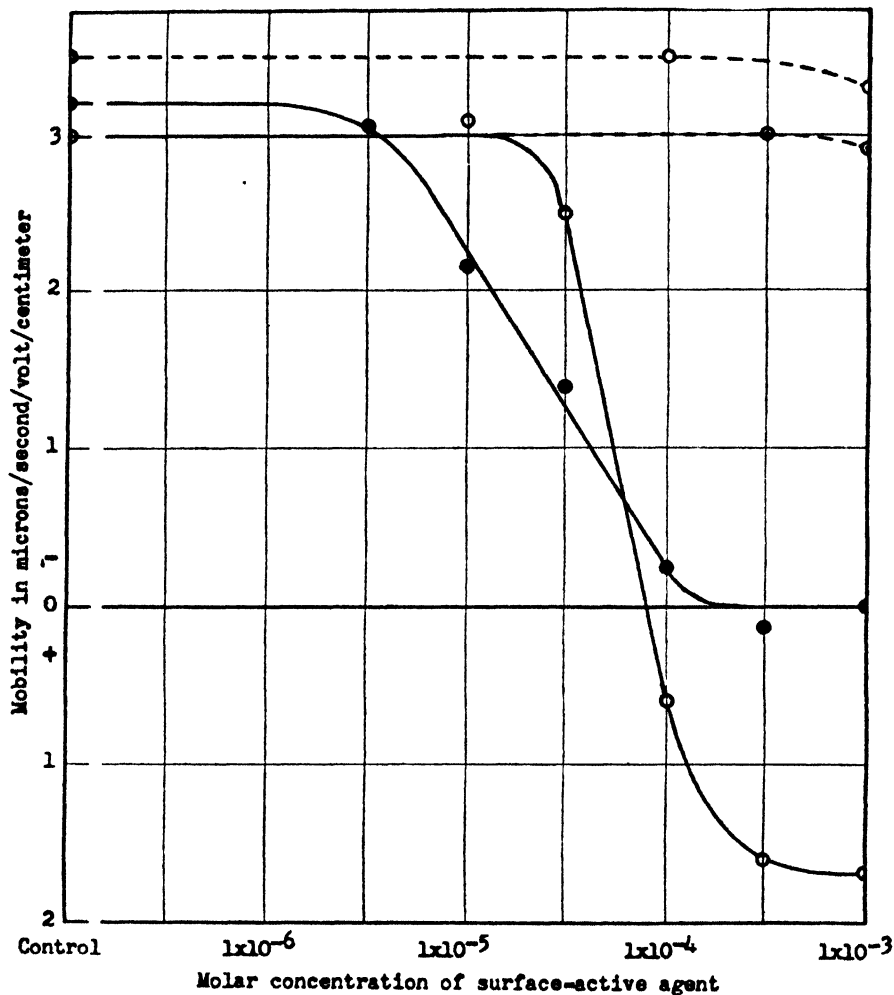


FIG. 5. MOBILITY-CONCENTRATION CURVES FOR *BACILLUS PSEUDOTETANICUS*

Vegetative cells, ○ — ○ with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate; spores, ● — ● with cetyl pyridinium chloride, ○ — — ○ with sodium tetradecyl sulfate.

photomicrographs give no indication of a waxy layer at the surface of *Mycobacterium tuberculosis*. Likewise, Knaysi (1929) has presented microchemical evidence that no such waxy capsule surrounds the cells of *Mycobacterium tuberculosis*. Freund (1925) suggested that acid-fast bacteria have a protein surface, because their agglutination reactions are similar to those of particles of protein.

The experiments performed with *Mycobacterium smegmatis* and *Mycobacterium phlei* are recorded in figure 6. The curves for the two species resemble each other but differ markedly from the curves obtained with the other bacteria investigated.

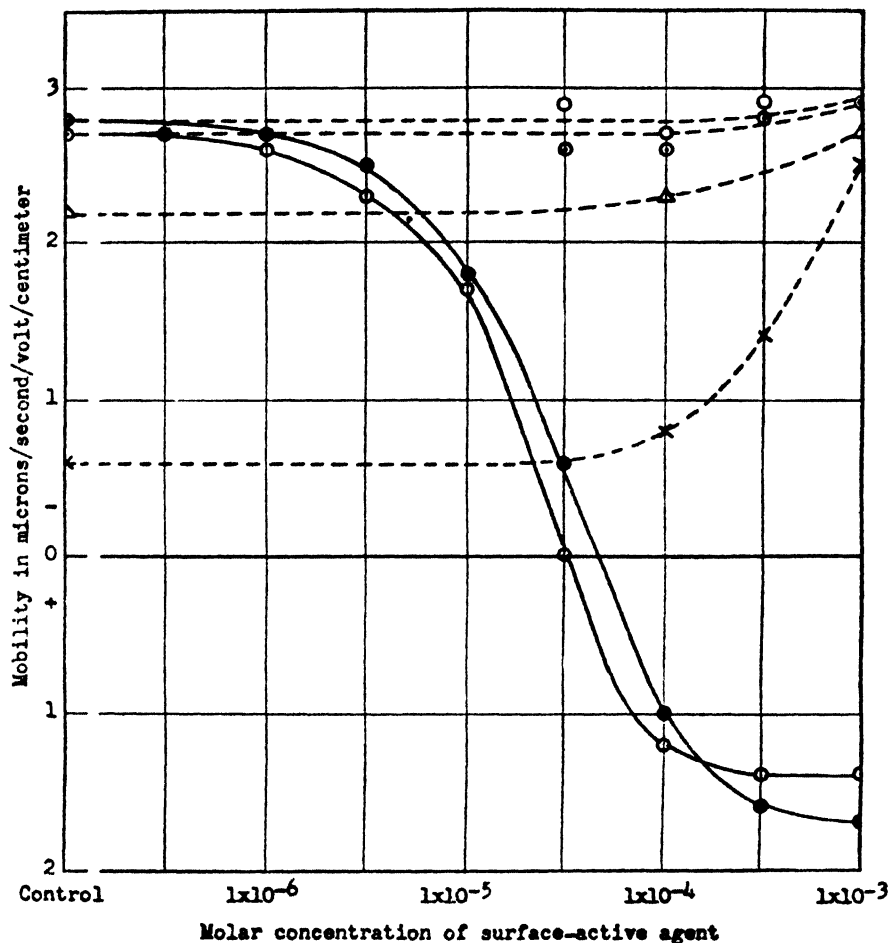


FIG. 6. MOBILITY-CONCENTRATION CURVES FOR MYCOBACTERIUM SMEGMATIS AND MYCOBACTERIUM PHELI

Mycobacterium smegmatis, \bigcirc — \bigcirc with cetyl pyridinium chloride, \bigcirc — \bigcirc with sodium tetradecyl sulfate at pH 6.9, \triangle — \triangle at pH 5, and \times — \times at pH 4; *Mycobacterium phlei*, \bullet — \bullet with cetyl pyridinium chloride, \bigcirc — \bigcirc with sodium tetradecyl sulfate.

The mobility with the cationic wetting agent begins to decrease in a very dilute solution, 1×10^{-6} M. The change in mobility is more gradual than it is with any of the other bacteria studied, occurring over a very wide range of concentration. A high positive charge is attained.

The mobility is sensitive to changes in pH. Mobility-concentration curves

with the anionic wetting agent were obtained at pH 7, 5, and 4. At pH 7 the mobility with STS increased only slightly, and at pH 5 a little more. At pH 4 it increased markedly, the slope increasing as the concentration of wetting agent increased. The mobility attained with 1×10^{-3} M STS at pH 4 approximates that with the same concentration at pH 5 and 7.

Chondrococcus columnaris

Chondrococcus columnaris, which was isolated by Ordal and Rucker (1944) and shown to be the causative agent of a fatal disease in salmon, was selected as a representative of the Myxobacteriales. Because of the differences between myxobacteria and true bacteria, this species was of special interest to study electrophoretically. The results are plotted in figure 7.

The change in mobility after treatment with STS is very striking. The mobility increases tremendously, more than tripling its value. The mobility of *Spirillum volutans* and *Mycobacterium smegmatis* also increased when the initial mobility was adjusted to a low value by lowering the pH; in these cases, however, the mobility did not increase nearly so much, and the change began at a higher concentration of the wetting agent.

Chondrococcus columnaris forms microcysts which, although representing a resting stage, are not comparable to bacterial spores, because they do not possess much resistance to injurious agents. The electrophoretic mobilities of the microcysts are shown in figure 7. The curves for the microcysts are similar to those for the vegetative cells except that the increase in mobility after treatment with STS is less and begins at a greater concentration.

The same strain was reinvestigated about 18 months later. During this time it had been transferred on tryptone media and had lost its pathogenicity for salmon. The character of its growth in tryptone broth had changed from a uniform turbidity to a slimy mass from which it was difficult to separate all the cells, even with repeated washings. Although the initial charge of these cells was approximately the same as before, the behavior of the cells after treatment with STS was very different. The mobility of a few cells increased in the presence of the highest concentrations of STS, though only a fraction as much as had the original culture, and most of the cells showed no change in mobility.

Another strain freshly isolated from diseased squaw fish, but not pathogenic to salmon, was investigated. The cells from this culture were easily suspended after washing. The results are also shown in figure 7. The behavior of this strain on treatment with both surface-active agents differs considerably from that shown by the original culture pathogenic to salmon. The initial charge is lower and on treatment with STS increases slightly at a 10^{-3} M concentration. On treatment with CPC the charge is reduced to zero and no positive charge results.

It seems reasonable to believe that with the original culture from salmon the high mobility in the presence of STS and the reversal of charge and mobility in the presence of CPC are due to the presence of material in the surface, presumably lipid, in which the hydrocarbon groups of the surface-active agents are soluble.

This material has apparently disappeared during the period of laboratory culture and is not present in the culture of low virulence obtained from squaw fish. An attempt is now being made to build up the pathogenicity for salmon of the original strain of *Chondrococcus columnaris* in order to determine whether the original surface properties will be restored.

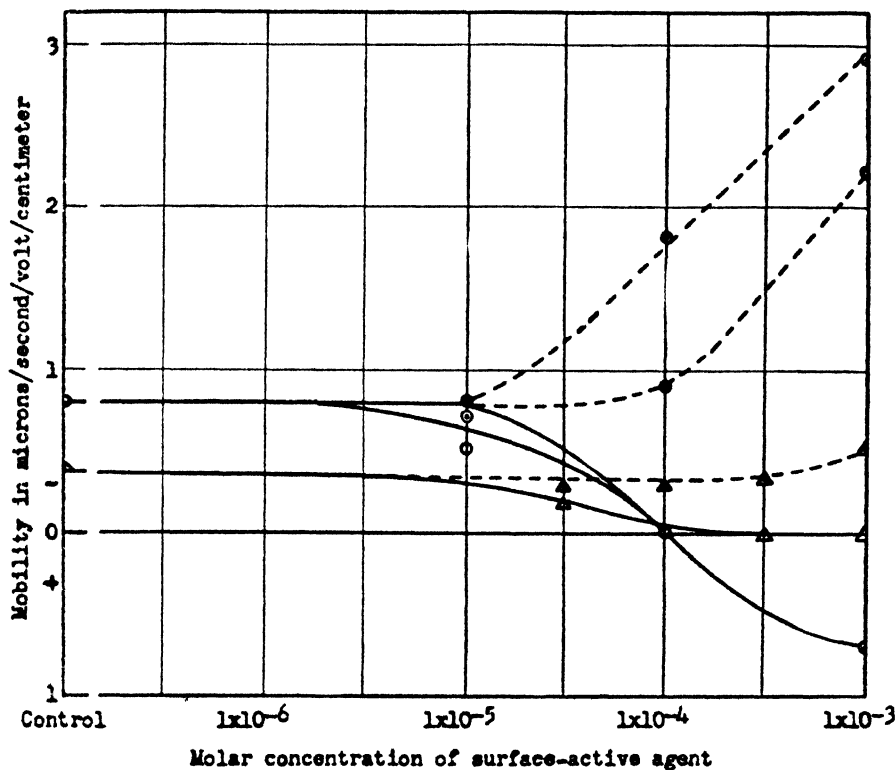


FIG. 7. MOBILITY-CONCENTRATION CURVES FOR *CHONDROCOCCUS COLUMNARIS*

Chondrococcus columnaris (salmon) vegetative cells, ○—○ with cetyl pyridinium chloride, ○—○ with sodium tetradecyl sulfate; cysts, ●—● with cetyl pyridinium chloride, ●—● with sodium tetradecyl sulfate; *Chondrococcus columnaris* (squaw fish), △—△ with cetyl pyridinium chloride, △—△ with sodium tetradecyl sulfate.

Spirochaeta sp.

A free-living species of *Spirochaeta*, isolated in pure culture from a hydrogen sulfide spring, was investigated as a representative of the spirochetes. Although this organism, which is 0.9 to 1.2 by 10 to several hundred microns, is much larger than the pathogenic spirochetes, yet it resembles them in some respects such as requiring complex media for growth in pure culture.

The electrophoretic mobilities after treatment with surface-active agents are plotted in figure 8. They are unlike those obtained for any of the other bacteria studied. The values for the mobility were variable, possibly because the spirochetes are so long that it is impossible to make a reading with the entire cell at

the stationary level. With anionic STS the mobility increases slightly. The mobility is very sensitive to change with CPC, beginning to decrease at 1×10^{-6} M. It decreases gradually and acquires only a very low positive charge. The mobility was 0.7 microns per second per volt per centimeter at pH 4, 1.1 at pH 5, and 1.3 at pH 6.9. The cells of *Spirochaeta* exhibit polarity in the electric field. Although most bacteria are so influenced by Brownian movement that it is impossible to determine whether they show this property, large cells

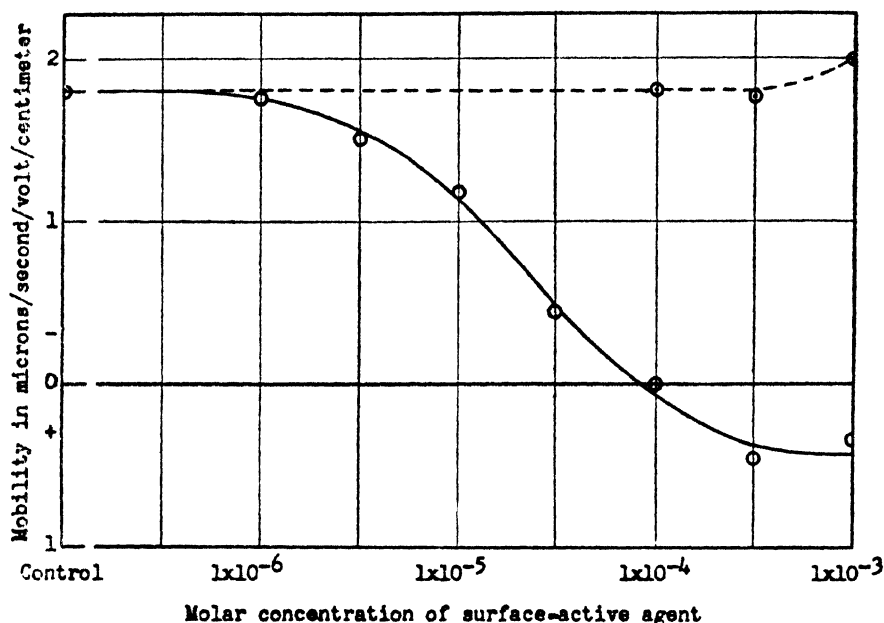


FIG. 8. MOBILITY-CONCENTRATION CURVES FOR SPIROCHAETA SP.

○—○ with cetyl pyridinium chloride, ○— —○ with sodium tetradecyl sulfate.

of a species of *Bacillus*, as well as cells of *Spirillum* and *Spirochaeta*, have been observed to do so.

SUMMARY AND DISCUSSION

The experimental work reported in this paper is exploratory in nature and represents an attempt to establish a method of characterizing bacterial surfaces. A variety of bacteria have been treated with an anionic and a cationic surface-active agent, giving concentration-mobility curves which are distinctive for the various bacterial species. With most bacteria the shapes of the concentration-mobility curves have remained consistent when run at widely different times, except for small changes in character such as initial charge. In other cases, particularly with *Escherichia coli* and *Chondrococcus columnaris*, there have been marked changes in the character of the curves over a period of time. In these cases, dissociation is probable, though not necessarily recognizable by ordinary methods.

In general, treatment with sodium tetradecyl sulfate has produced no change,

a slight change, or a marked change in electrophoretic mobility. If a change occurs, the concentration of STS at which this first occurs varies considerably.

Treatment with the cationic surface-active agent, cetyl pyridinium chloride, yields a general pattern of decrease of charge, reversal of charge, and stabilization of charge. The degree of these changes and the concentration of CPC at which they occur are very different among the bacteria studied. Bacteria treated with CPC differ especially in the concentration at which the first change in mobility occurs, the rate of change of mobility, the concentration at which the charge is reversed, the magnitude of the positive mobility acquired, and the concentration at which the positive mobility is stabilized.

The changes in mobility which occur on treatment with surface-active agents must depend both on the chemical nature of the surface-active agent and that of the cell surface. The cell surface involved is, of course, the outermost layer under the particular experimental conditions. In some cases this may be the slime layer or capsule. If the slime layer is absent or has been removed by the experimental procedure, it is the cell wall.

The selection of sodium tetradecyl sulfate and cetyl pyridinium chloride for this investigation was somewhat fortuitous, and it is to be expected that more distinctive concentration-mobility curves will be obtained by the use of surface-active agents of different structures or by the use of other polar molecules. Evidence of this has been obtained in electrophoretic investigations of particles of known composition using surface-active agents of varying structure. Bradbury and Jordan (1942) have measured the variation of electrophoretic mobility with time when suspensions of *Escherichia coli* in equilibrium water were exposed to *p*-aminobenzoic acid, sulfanilamide, and related chemical compounds. Active antibacterial compounds produced characteristic mobility-time curves, whereas inactive substances produced an initial change in mobility which did not alter with the time of contact. The conclusion was drawn that sulfanilamide and related drugs behaved like *p*-aminobenzoic acid at the bacterial surface. It was concluded further that the association of the drug with the organism is a function of the aromatic amino groups, and that the polarity produced by the resonance of active molecules is one of the factors determining activity. Although Abramson (1934) and Moyer (1940) have severely criticized the use of suspensions of bacteria in distilled water, the data of Bradbury and Jordan are convincing, and their method may prove useful for the study of bacterial surfaces.

The method described in this paper will prove of value in the determination of the chemical nature of substances present at the cell surface only if the presence of these substances is characteristically reflected in the electrokinetic behavior of bacteria in the presence of surface-active agents or other reagents. A beginning has been made in the determination of the electrokinetic behavior of particles of known composition in the presence of varying concentrations of surface-active agents. Striking differences have been observed in the electrophoretic behavior of substances such as hydrocarbons, protein, phospholipid, cholesterol, and inert surfaces in the presence of surface-active agents. These results will be reported separately. Proteins such as lipoproteins, nucleoproteins, mucins,

and the ribonucleate-protein complex reported by Henry and Stacey (1943) and by Bartholomew and Umbreit (1944) to be present in the outer layer of gram-positive organisms should be investigated, as well as chitin, cellulose, phospholipids, the waxes associated with acid-fast bacteria, polysaccharides, and the lipopolysaccharides from enteric bacteria.

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THE ANTIGENIC COMPLEX OF SHIGELLA PARADYSENTERIAE, BOYD TYPE P274

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Boyd (1940) in his description of the dysentery organisms distinguished several serological types which had the general biochemical characteristics of Flexner organisms but which showed practically no serological relationships to that group, to other *Shigella* types, or to one another. Among the few exceptions to this was type P274, which was related by a common antigen to *Shigella alkalescens*. This component has been identified as the "C," or related to the "C," antigen of *S. alkalescens* which is found also in other genera of the Enterobacteriaceae including normal *Escherichia* (Stuart *et al.*, 1943; Wheeler, 1944).

During the past two years a considerable number of cultures have been isolated from cases of diarrhea and from carriers and studied for their relationship to the P274 antigen complex. The results seem of general interest and are herewith reported, since they show (1) antigenic differences between certain type P274 strains; (2) a variety of biochemical types which are related to type P274 cultures; and (3) the serological complexity of the antigen of type P274.

METHODS AND SOURCE OF CULTURES

The majority of strains were isolated from cases of diarrhea or from food handlers in the Mediterranean area during 1943 and 1944. Included also were typical *S. alkalescens* strains as well as coliform cultures possessing *S. alkalescens* antigens which have been described by Stuart *et al.* (1943). Immunization, agglutination, and adsorption methods were essentially the same as those previously reported (Wheeler, 1944).

BIO-TYPES

A considerable number of strains with varying biochemical reactions were found to be related to *Shigella paradysenteriac*, type P274. Representative strains are shown in table 1. Carbohydrate fermentations were recorded after 3 weeks' incubation at 37 C. Bromeresol purple indicator was used in the broth medium. Tests for acetylmethylcarbinol were made by the Barritt modification of the Voges-Proskauer test. Kovac's reagent was used for indole tests, and trimethylamine oxide reduction tests were done after 24 hours' incubation by the method of Wood and Baird (1943). Motility was determined both in broth and 0.3 per cent agar after room temperature incubation.

Included are two cultures of type P274 (563, 2084). These were identical in biochemical reactions. Among some 43 strains of sero-type P274 the only varia-

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tion in fermentation reactions occurred with maltose and dulcitol. About one third of the strains failed to attack maltose in 3 weeks, whereas the remainder produced an acid reaction in from 2 to 21 days. About 20 per cent of type P274 cultures fermented dulcitol in from 5 to 21 days.

Culture 2439, an anaerogenic paracolon, was the only strain of this type encountered. The next four cultures listed in table 1 (120, ER, 2373, ME) had identical somatic antigens. Strain ER differed from strain 120, a typical *S.*

TABLE 1
Biochemical reactions of cultures with antigens of S. paradysenteriae, type P274

CULTURE	GLUCOSE	LACTOSE	SUCROSE	SALICIN	MALTOSE	MANNITOL	RAMNOSE	ARABINOSE	DULCITOL	XYLOSE	SORBITOL	CITRATE	V.P.	INDOLE	T.M.A.	MOTILITY
563 (type P274)...	A	0	0	0	A*	A	0	A	0	0	0	0	0	0	0	0
2084 (type P274)...	A	0	0	0	A*	A	0	A	0	0	0	0	0	0	0	0
2439.....	AG	A*	0	0	A	A	A	A	0	A	A*	0	0	+	+	0
120 (<i>S. alkalescens</i>)	A	0	0	0	A	A	A	A	A	A	A	0	0	+	+	0
ER.....	A	0	0	0	A	A	A	A	A	A	A	0	+	+	+	0
2373.....	A(G)	A*	0	A*	A	A	A	A	A	A	A	+	0	+	+	+
ME.....	A	A*	A*	0	A	A	A	A	A	A	A	0	0	+	+	0
2375.....	A	A	A	0	A	A	0	A	0	A	A	0	0	+	+	0
2437.....	AG	0	0	0	A	A	0	A	A	0	A	0	0	+	+	0
2370.....	A	0	0	0	0	A	0	A	0	0	A	0	0	+	+	0
2372.....	A	0	0	0	A*	A	A	A	A*	A*	A	0	0	+	±	0
2440.....	AG	0	0	0	A	A	A	A	A	A	A	0	0	0	+	0

A = acid; AG = acid and gas; A* = acid reaction delayed 48 hours or more; 0 = no reaction.

All cultures were negative with inositol and urea.

* Transitory positive reaction, disappeared after a few transplants on agar.

alkalescens, only in giving a positive Voges-Proskauer reaction when first isolated. This property was lost after a few transfers. The other two strains are included since they are new bio-types of *S. alkalescens* and enlarge the series of bio-types which have already been shown for this sero-type (Stuart *et al.*, 1943, 1944). Strain 2373 utilized citrate and was motile. Strain ME fermented both lactose and sucrose. The lactose-and-sucrose-fermenting variant arose as a daughter colony in the colorless parent colony on eosin-methylene-blue agar. The original plating showed numerous colonies with daughter papillae. Subsequent platings showed fewer papillae, and they were absent on the fourth plating. Of the atypical biochemical strains of *S. alkalescens* previously (Stuart *et al.*, 1943) and herein reported, only the ME strain produced variants through daughter colonies.

In addition, lactose-fermenting and motile strains of *S. alkalescens* were encountered similar to ones already described (Stuart *et al.*, 1943).

Two cultures were found, 2375 and 2437, which had the "C" fraction of *S. alkalescens* and the complete somatic antigens of the coli-4 culture (Stuart *et al.*, 1943). These are new bio-types of this sero-type and for this reason are included in table 1. Strain 2370 and another biochemically and serologically identical culture, 2405, had the biochemical reactions of *S. paradysenteriae*. Trimethylamine oxide was reduced, however, in contrast to all *S. paradysenteriae* strains except type P143.

Five strains have been studied which are identical with culture 2372 shown in table 1. Another culture, 2440, differing in gas production and in indole reaction was closely related serologically to culture 2372, but was not identical with it. Strain 2372 is the one which Neter (1944) has called "*S. alkalescens* sero-type III." The biochemical reactions of these strains were similar to those of *S. alkalescens* except for trimethylamine reactions, which were variable. All strains reduced the oxide, although some very weakly.

TABLE 2
Agglutination reactions of cultures related to S. paradysenteriae, type P274

ANTISERUM	AGGLUTININ TITER WITH CULTURE						
	563	2084	2439	120	2375	2370	2372
563	2,560	2,560	2,560	2,560	1,280	0	0
2084	2,560	2,560	2,560	2,560	2,560	320	2,560
2439	320	160	40,960	0	0	0	0
120	160	80	0	10,240	320	0	0
2375	320	160	80	320	20,480	0	160
2370	0*	20	0	0	0	2,560	160
2372	320	40	20	0	40	320	40,960

* Titer of less than 1:20.

SEROLOGICAL RELATIONSHIPS

Direct agglutination reactions of the serologically different strains are shown in table 2. Only a single strain of each sero-type is included. The identities of the other cultures of each group were established either by reciprocal adsorptions or, when antisera were not prepared, by adsorption of the serum listed in the table.

A serological difference was found between certain type P274 antisera. This is readily shown by the reactions of antisera prepared from strains 563 and 2084 with cultures 2370 and 2372. The difference in reactivity between the two sera is not a peculiarity of these two immunizing strains, since six antisera have been prepared from type P274 strains and three of each kind were obtained. The antigenic difference is not apparent in the reactions of cultures 563 and 2084 shown in table 2, however, and at the time those tests were made practically no differences could be shown. When first isolated, strain 2084, and

others like it, could be distinguished by their strong agglutination in *S. alkalescens* antiserum.

The serological relationship of all the strains in table 2 to *S. paradysenteriae*, type P274, is shown through the reactions of serum 2084. The reciprocal tests

TABLE 3

Antigenic components related to S. paradysenteriae type P274 as shown by adsorption

ANTISERUM	ADSORBED WITH	AGGLUTININ TITER WITH CULTURE						
		563	2084	2439	120	2375	2370	2372
563 (P274)	2084	0-80	0	0	0	0		
	2439	1,280	1,280	0	2,560	640		
	120	1,280	1,280	2,560	0	160		
	2439 & 120	1,280	1,280	0	0	80		
	2375	1,280	2,560	2,560	2,560	0		
2084 (P274)	563	0	0	0	0	0	0	0
	2439	1,280	1,280	0	2,560	1,280	80	5,120
	120	1,280	1,280	2,560	0	160	160	5,120
	2375	1,280	1,280	2,560	1,280	0	20	2,560
	2370	1,280	1,280	2,560	2,560	2,560	0	2,560
	2372	1,280	1,280	2,560	2,560	1,280	0	0
2439	563	0	0	20,480				
120 (<i>S. alkalescens</i>)	563	0	0		10,240	160		
	2375	160	80		10,240	0		
2375	563	0	0	20	80	10,240		20
	120	80	80	40	0	10,240		20
2370	2372						1,280	0
2372	563	0					160	20,480
	2370	80					0	20,480
	573 (Boyd P143)	320					320	40,960
	2214 (<i>S. dispar</i> I)	320					320	40,960
	2213 (<i>S. dispar</i> II)	0					0	0

of type P274 cultures gave weaker titers in most instances, e.g., 80 to 160 for antiserum 120 (*S. alkalescens*); 160 to 320 for 2439 and 2375 antisera; and insignificant reactions with antiserum 2370. With the remaining cultures shown in table 2 reciprocal agglutination tests indicate that the principal antigens are

type specific and that the cross reactions are due to other minor antigens. In only one case was the heterologous titer as much as 12.5 per cent of the homologous titer.

The relationships within this group of cultures are further illustrated by the selected adsorption tests summarized in table 3. No differences between the two type P274 strains could be shown by reciprocal adsorptions. The ability of strain 563 to remove from antiserum 2084 agglutinins for cultures 2370 and 2372 is surprising, since these antibodies were not produced by immunizing with strain 563. On the other hand, adsorption of 563 serum with 2084 culture on some occasions failed to remove all homologous agglutinins although the maximum titer was never greater than 1:80. It must be assumed that antigenic change has occurred in the 2084 strain between the time of immunization and the test, at least in so far as it is possible to show antigens *in vitro*.

The fraction shared between culture 2439 and type P274 is distinct from components shared with other cultures, since adsorption of either type of P274 antiserum with culture 2439 failed to remove agglutinins except for the adsorbing strain; likewise with culture 2375. Both 120 (*S. alkalescens*) and strain 2375 share an antigen with type P274. This is not the complete "C" fraction of *S. alkalescens*, which was defined as the common antigen of coli-4 and *S. alkalescens* (Stuart *et al.*, 1943), since adsorption of P274 antisera with *S. alkalescens* failed to remove completely the agglutinins for coli-4 (2375) and culture 2375 failed to take out the agglutinins for *S. alkalescens*. Adsorptions of 120 and 2375 antisera are corroborative.

A related specificity is shown by strains 2370 and 2372 since adsorption of antiserum 2084 with 2372 culture removed antibodies for culture 2370 as well as for itself. With the exception of the last one listed, which will be discussed subsequently, the remaining adsorptions in table 3 illustrate the complexity of specificities exhibited within this group of cultures.

When the relationships of these organisms to other members of the *Shigella* group were studied by reciprocal agglutination tests, certain reactions occurred of sufficient strength to deserve mention. Culture 2372 was most interesting in this respect since it was agglutinated by antisera prepared against (1) *S. paradysenteriae*, Boyd type P143, to within one dilution of the homologous titer, (2) *Shigella dispar* sero-type II (Carpenter, 1943, 1945), to within one or two dilutions of the homologous titer, and (3) most Flexner types. Adsorption of type P143 antiserum by 2372 culture failed to reduce the homologous titer, and a major common antigen distinct from the specific P143 antigen must be postulated between type P143 and strain 2372. The reciprocal adsorption likewise failed to decrease the homologous titer, as shown in table 3. Adsorption of one *S. dispar* type II antiserum with culture 2372 decreased the titer for *S. dispar* from 20,480 to 640, and the homologous agglutinins were completely removed from another *S. dispar* type II antiserum. The reciprocal adsorption of 2372 antiserum with *S. dispar*, type II, completely removed agglutinins for strain 2372 (table 3). Culture 2372, therefore, appears to be very closely related to some, and antigenically identical with other, type II *S. dispar* strains. Agglu-

tionation by Flexner antisera occurred only to titers of 1:160 to 1:320, and the 2372 antiserum agglutinated *S. paradysenteriae*, Flexner Y, 1:320, type IV 1:80, type VI 1:80, and "B. wakefield" (Berger, 1945) 1:5,120. The culture apparently has some of the Flexner group antigen, but it appears as a minor component.

Another significant cross reaction was encountered between *S. alkalescens* and *S. dysenteriae* (Shiga). This will be discussed in more detail elsewhere (Wheeler and Stuart).

DISCUSSION

The cultures described make up a series of related types which in many respects is analogous to that found for *S. alkalescens* (Stuart *et al.*, 1943). A species-specific antigen for type P274 that is analogous to the "A" antigen of *S. alkalescens* can be shown by the agglutinin in P274 antiserum (563) following a combined adsorption with cultures 120, 2375, and 2439. A fraction is shared with strain 2439, a "paracolon" organism, analogous to the *S. alkalescens* "B" antigen. A third component present in type P274 and *S. alkalescens* cultures is shared by certain coliform cultures (2375) and is actually related to, or is a part of, the *S. alkalescens* "C" antigen. Finally, a difference can be shown between type P274 strains; for example, those resembling culture 563 and those like strain 2084. This is similar to the variation of the "D" antigen among *S. alkalescens* strains.

The 2084 strain of type P274 does not appear to be stable as in 10 months since isolation it changed so that in serological reactions it was indistinguishable from strain 563. The differences, however, are readily apparent in the two antisera. The reactions of antiserum 2084 are important since they relate the other organisms in the group.

Strain 2372 is an important connecting link in the *Shigella* group since it has the biochemical reactions of *S. alkalescens*; but none of the *S. alkalescens* is antigenically closely related to or identical with *S. dispar* type II, exhibits a close serological relationship to *S. paradysenteriae* Boyd type P143, has a part of the group antigen of the Flexner organisms, and is related to Boyd type P274 through strong reactions in antiserum 2084.

Strains like 2372 afford an example of the difficulties of classification in the *Shigella* group. Even though the organism has the biochemical reactions of *S. alkalescens*, in view of the absence of serological connection to *S. alkalescens* and since it is so closely related to other *Shigella*, there seems little justification for classifying this organism as *S. alkalescens* as has been done (Neter, 1944). On the other hand, the relatively weak agglutination by Flexner sera (1:320) and the reciprocal agglutination of Flexner Y in 2372 antiserum (1:320) when the homologous titers are 10,240 to 20,480 do not appear to be sufficient evidence to place this organism with the Flexner group. The closest serological relationship is to *S. dispar*, sero-type II (Carpenter, 1943, 1945), yet it differs biochemically from *S. dispar* in the failure to ferment lactose.

The pathogenic significance of this type is not known, since the 5 strains were isolated from civilian food handlers whose history was not available. The original source of the aerogenic serologically related organism 2440 (table 1) is not known.

Strain 2370 and an identical culture, 2405, fall into the paradysentery group with respect to biochemical characteristics, yet the only demonstrable serological relationship to that group is through antiserum 2084. A minor component was shared with the 2372 strain. The history of one of these strains is not available; the other was isolated from a food handler. On the basis of information at hand these strains constitute another serological type of *S. paradysenteriae*.

The cultures of this whole series, especially strain 2372, illustrate the variety of specificities which can be demonstrated for the antigenic mosaic of a single strain. Presumably the antigenic specificity as shown by agglutination procedures depends on the surface-reacting groups of the antigen, and cross reactions depend on similarities of surface groupings rather than on distinct complete antigen fractions. Insofar as the diagnostic laboratory is concerned, either concept provides the same working basis for identification since by suitable adsorption procedures the "type-specific" major fractions can be identified. The presence of related specificities, however, among biochemically different cultures is further data to be added to much that has recently accumulated pointing toward the concept that the enteric group of bacteria is a continuous series of intergrading types.

SUMMARY

A series of cultures serologically related to *Shigella paradysenteriae* type P274 has been described which shows (1) serological differences between type P274 strains; (2) a common antigen between type P274 and a slow lactose-fermenting gas-producing paracolon organism; (3) a serological relationship to *S. alkalescens* and other serologically identical but biochemically different organisms, including an acetylmethylcarbinol producer; (4) serological relationship of type P274 to new bio-types of a previously described (Stuart *et al.*) sero-type of coliform organism; (5) serological relationship to a new sero-type of *S. paradysenteriae* (strain 2370); and (6) serological relationship to an organism of *S. alkalescens* bio-type, but not sero-type, closely allied serologically to *S. dispar*, *S. paradysenteriae* type P143, and also less closely related to the Flexner group.

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FURTHER OBSERVATIONS ON THE NUCLEAR MATERIAL OF THE BACTERIAL CELL

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The literature on the existence and state of a nucleus in the bacterial cell has been repeatedly reviewed. Among the most recent reviews are those of the author (1938, 1944), Delaporte (1939-1940), and Lewis (1941).

In 1912, the author, using a number of morphological and microchemical criteria, demonstrated a nucleus in the cell of *Staphylococcus flavo-cyanus*. This was confirmed by Knaysi and Mudd (1943), who observed the granules with the electron microscope, showed their probable ability to divide, separated them from the cell by sonic vibrations, and demonstrated that they contained thymonucleic acid. In extending their work to other species, Knaysi and Mudd observed granules of possibly nuclear nature in a meningococcus but were unable to find such granules in a number of other species, rods or cocci, including a strain of *Streptococcus pyogenes* known to contain, relatively, considerable amounts of thymonucleic acid (Sevag, Smolens, and Lackman, 1940). They concluded that in certain species or strains the nuclear material may be in the diffuse state.

Following up his work with *Staphylococcus flavo-cyanus*, the author attempted to apply the same methods to young cells of several of the aerobic sporeformers. It is true that certain procedures gave pictures similar to those interpreted by some as showing nuclei, but our long familiarity with the structure and behavior of the bacterial cell gave us sufficient ground to conclude that the results were negative.

During a recent study of the cytology of endospore formation in strain C₃ of *Bacillus cereus* (1946b), it was possible, under definite conditions, to predict sporulation within a relatively short time. Consequently, microcultures at various stages of development were subjected to the Feulgen reaction: the microcultures were disconnected; the cover glass was cautiously removed, allowed to dry, cleaned with xylol, and dropped in 95 per cent alcohol. At the end of 2 days, the cover glass was taken out of the alcohol, rinsed with distilled water, hydrolyzed in N HCl for 10 minutes at 60 C, placed in the Schiff reagent for 6 hours, put in a sulfurous bath for 5 to 10 minutes, rinsed with distilled water, and dried; the film of cells was then examined in water or in sulfurous water. The Schiff reagent and sulfurous bath were prepared in accord with Delaporte's (1939-1940) formulae.

The results are illustrated in figures 1 to 3. Figure 1, which represents a microcolony undergoing sporulation, shows numerous strongly positive, homogeneous spots which represent forespores and immature endospores. Here and there, one finds spores which show a positive, internal rod or granule within a colorless zone. The colorless zone is surrounded by a positive line or halo. On



FIG. 1. *BACILLUS CEREUS*, C_4

One-day-old microculture at 25 to 28 C, prepared by mixing a 2-week old culture in VFC¹ with an equal volume of MITG² and depositing a minute quantity of this mixture on a droplet of 1.5 per cent bacto agar, etc. (Knaysi, 1940). Feulgen reaction. Note the numerous homogeneous, black spots representing forespores and immature spores. In view of the fact that endospores are not simultaneously formed in a colony, a number of differentiated mature endospores are also seen.

FIG. 2. *BACILLUS CEREUS*, C_3

One-day-old microculture similar to that of figure 1. Feulgen reaction. Note both immature (left) and mature (right) endospores.

FIG. 3. *BACILLUS CEREUS*, C_3

A three-day-old microculture at 25 to 28 C, prepared as in figure 1. Feulgen reaction. Note absence of homogeneous endospores. Mature endospores show a Feulgen-positive central rod or granule inside a colorless zone, which is surrounded by a strongly positive halo.

FIG. 4. SCALE FOR ALL ILLUSTRATIONS

¹ VFC = vitamin-free casein hydrolyzate

² MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose.

account of the small number in young colonies, these differentiated spores must be mature ones. Homogeneous and differentiated spores are also found in figure 2, which also represents a colony undergoing sporulation. Microcolonies in which sporulation has long been completed (figure 3) contain differentiated spores only, a final proof that these represent mature endospores. The cytoplasmic membrane and the lipoprotein granules, which are usually formed in the cytoplasmic membrane and eliminated into the cytoplasm before sporulation (Knaysi, 1946a), are the only other differentiated structures which are Feulgen-positive.

DISCUSSION

The results summarized above show that there is no chromatin granule enclosed in the forespore as was reported by Meyer (1897, 1899) for *Bacillus asterosporus* and *Bacillus tumescens*. The existence of such a granule was not revealed either by following the formation of the endospore (Knaysi, 1946b) or by application of the Feulgen reaction to the forespore and young endospore. On the contrary, the fact that the protoplasm of the forespore and young endospore gives a strong, homogeneous Feulgen reaction seems to indicate that its nuclear material is in a diffuse state and, *a posteriori*, that the nuclear material of the vegetative cell in the investigated strain is also in the diffuse state; this is supported by previous direct observation of the inclusion-free vegetative cell. It must also be added that Lewis (1934) was unable to detect a chromatin granule in the forespore of *Bacillus mycoides*.

The significance of the appearance of a Feulgen-positive rod or granule in the mature endospore is not entirely clear. It may mean that differentiation of a nucleus takes place during maturation of the endospore, or it may be the result of shrinkage of the protoplasm, which is known to take place during maturation (Knaysi, 1946b). Lack of knowledge about the structures of the endospore, their make-up, and their properties makes a final choice inadvisable, and one can argue for or against either possibility. In any case, the results of the present investigation support the conclusion of Knaysi and Mudd (1943) that bacteria differ with respect to nuclear differentiation. In certain species or strains, this differentiation takes place only under certain conditions of environment or of cultural development. In the known strains of organisms like *Staphylococcus flavo-cyaneus*, differentiation into nucleus and cytoplasm has become an established process.

SUMMARY

Microcultures of strain C₃ of *Bacillus cereus* were subjected to the Feulgen reaction at various stages of their development. In very young cultures, the only Feulgen-positive, differentiated structures are the cytoplasmic membrane and its extensions. At a later stage, cells grown aerobically form lipoprotein inclusions which are also Feulgen-positive. Forespores and young endospores stain homogeneously by the Feulgen procedure, but mature spores show a positive granule or rod. Whether the latter indicates differentiation or is due to

shrinkage of the protoplasm during maturation remains undetermined. The results show that, in the strain investigated, the nuclear material is diffuse both in the vegetative cell and in the young endospore, with a possible differentiation of a nucleus during maturation of the spore.

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THE ACTION OF PENICILLIN ON STAPHYLOCOCCUS¹

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Bacteria exposed to one of the active members of the sulfonamide group of drugs continue for a time to grow at a normal rate; growth is then interrupted and bacterial death begins (Hirsch, 1944). Some of the earlier work with penicillin indicated that, in contrast to this, penicillin causes the death of a certain proportion of the dividing cells of a growing culture without any appreciable lag period (Hobby *et al.*, 1942; Lee *et al.*, 1944). Rantz and Kirby (1944), however, have shown that at limiting dilutions of penicillin there may be a definite interval between the addition of penicillin and the onset of growth inhibition, as evidenced by turbidimetric determinations. From this, as well as from other studies of the action of penicillin, it has been concluded that the substance acts only on dividing cells, leaving nondividing cells unaffected. Hobby and Dawson (1944) likewise demonstrated that bacteria continued to multiply for a time after being planted in broth containing small concentrations of penicillin. According to these experiments, however, a similar lag in the onset of effective action of penicillin was also apparent when higher concentrations were used. The present studies were undertaken in order to gain more precise knowledge on this point and to determine how long the influence of penicillin on bacteria might last after its complete removal from the culture medium.

A commercial preparation of penicillin was used, the manufacturer's assay of its potency being accepted. The *Staphylococcus* employed was designated as strain Mx by Julianelle and Wiegand (1935) and is their type B. It is inhibited by 0.06, but not by 0.03, units of penicillin per ml and is thus somewhat less sensitive to penicillin than the Oxford strain. Tryptose phosphate broth and tryptose phosphate agar, manufactured by the Digestive Ferments Company, were used as culture media. Viable bacteria were determined by plate count after 24 hours' incubation. When samples were taken from the growing culture, they were chilled to 0 C in an ice bath after being withdrawn and held there until dilutions could be prepared for plating.

An initial series of experiments was set up to determine the effect of adding penicillin to a growing culture, according to the following pattern: To 10 ml of a 4-hour broth culture, penicillin solution was added in small volume and in amount sufficient to produce the desired concentration. At intervals thereafter a 0.5-ml portion was removed, immediately diluted to 5 ml with ice-cold saline solution, and centrifuged for 5 minutes at approximately 2,000 times gravity. The fluid was decanted, and the bacteria were resuspended in a second portion of saline solution (5 ml) and centrifuged. The organisms were then resuspended in cold saline solution and held briefly until dilutions could be prepared for plating. Preliminary experiments had indicated the necessity for

¹ This work was supported in part by a grant from the Ben Venue Laboratories, Inc.

removing the penicillin in this way, even though the final dilution in the plate might be well beyond the theoretically active concentration. The results are presented in a series of graphs (figure 1).

It is readily apparent that whereas the ultimate lethal effect of penicillin on the staphylococcal cells is uniform, the time of onset of the effect is distinctly influenced by the concentration of the drug. When the concentration is high (1.0 unit per ml), logarithmic growth is apparently replaced at once by logarithmic death. When the concentration is low (0.03 unit per ml), an increase in the population continues during the period of observation, but at a rate which is lower than normal. When the concentration causing "complete inhibition" in the overnight test was doubled, the phase of logarithmic death was preceded by a 30-minute period of apparently uninterrupted growth. These experiments

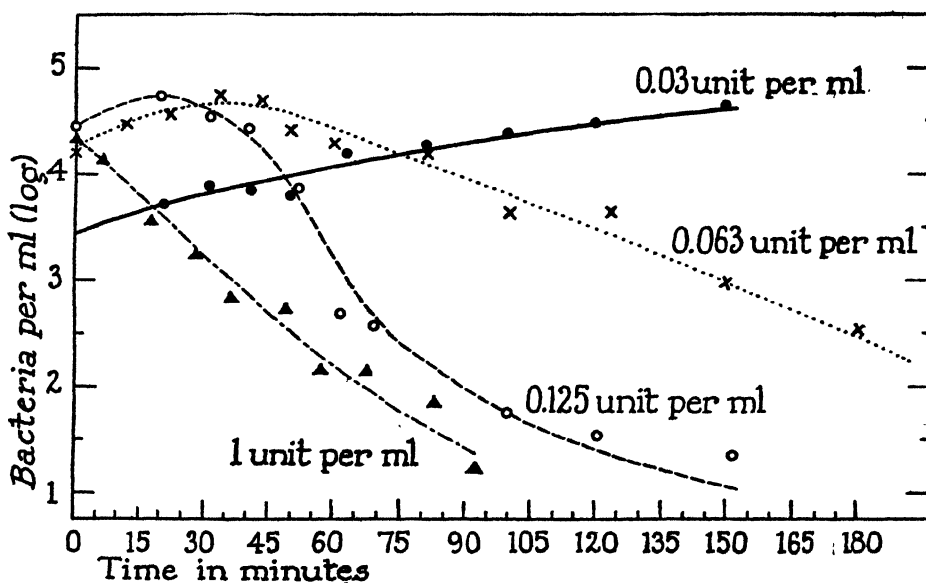


FIG. 1. CONTINUOUS EXPOSURE OF GROWING CULTURES TO 1.0, 0.125, 0.063, 0.03 UNITS OF PENICILLIN PER ML
Line drawn by inspection

were designed to follow the viable cell count only for the first period of penicillin action. In some experiments, evidence was obtained that the *rapid logarithmic* death rate continued only during the reduction of the population of the culture to 1 or 0.1 per cent of the maximum and that the rate was then reduced appreciably, in agreement with the findings of Hobby and Dawson (1944).

In a second series of experiments, penicillin was added to the liquid medium and after a predetermined time interval the bacterial cells were removed from the broth containing penicillin to a fresh, penicillin-free broth. The following experiment is typical of the series.

Fifty ml of broth (at 37 C) were seeded with 10^{-5} ml of an 18-hour broth culture of *Staphylococcus* Mx. After 4 hours a 12-ml sample was removed, penicillin was added to produce a concentration of 1.0 unit per ml, and incuba-

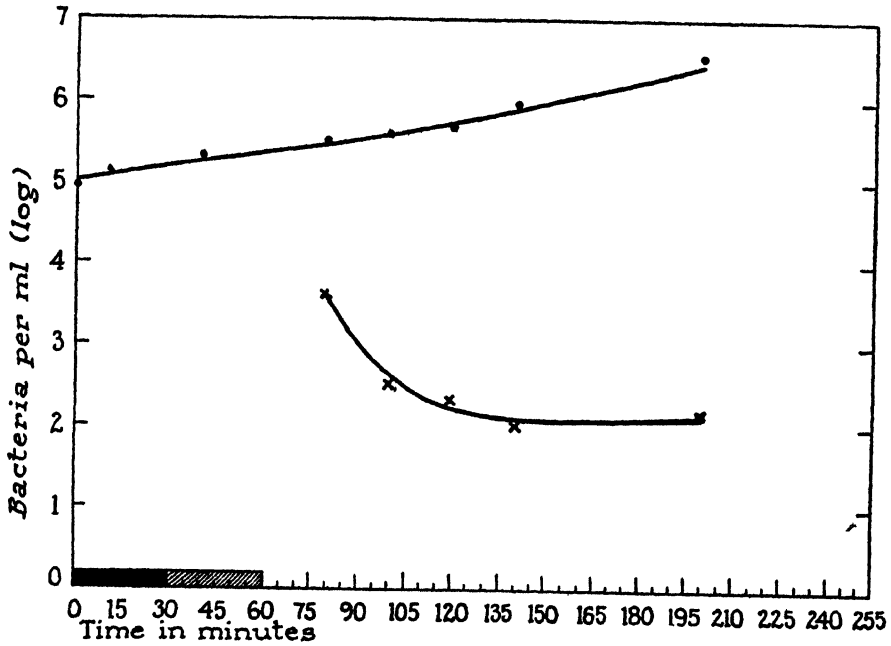


FIG. 2. EXPOSURE OF A GROWING CULTURE TO 1.0 UNIT PER ML OF PENICILLIN FOR 30 MINUTES

Upper curve, control culture. Lower curve, treated culture. Solid block indicates time of exposure; cross-hatched block, time of centrifugation and washing of culture. Lines were drawn by inspection.

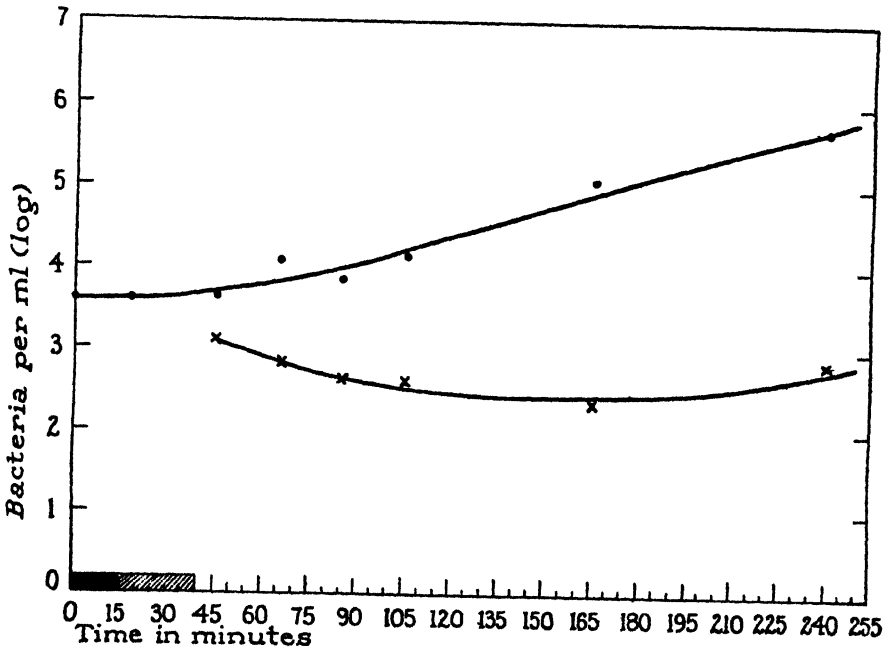


FIG. 3. EXPOSURE OF CULTURE TO 1.0 UNIT PER ML FOR 15 MINUTES

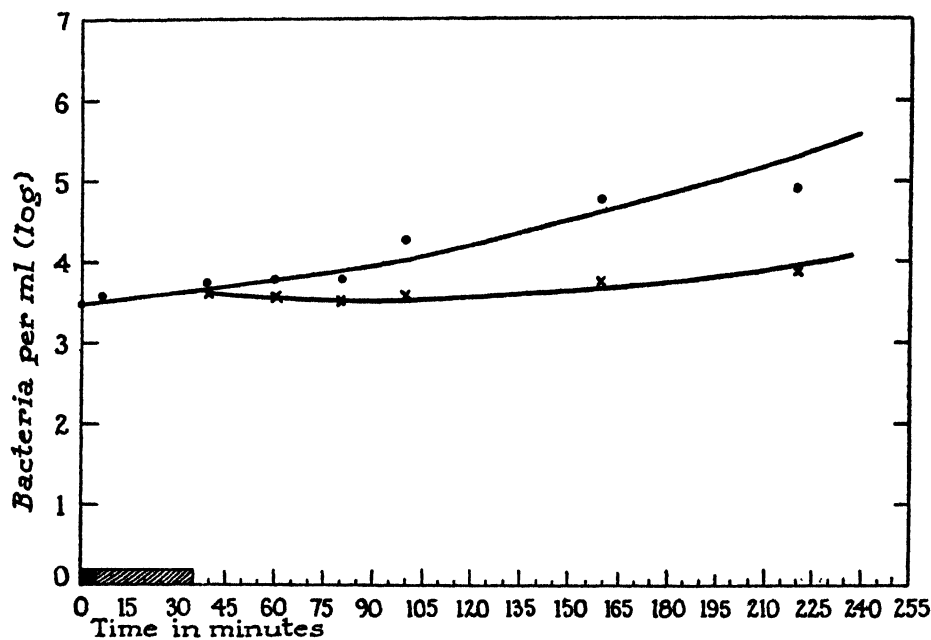


FIG. 4. EXPOSURE OF CULTURE TO 1.0 UNIT PER ML FOR 5 MINUTES

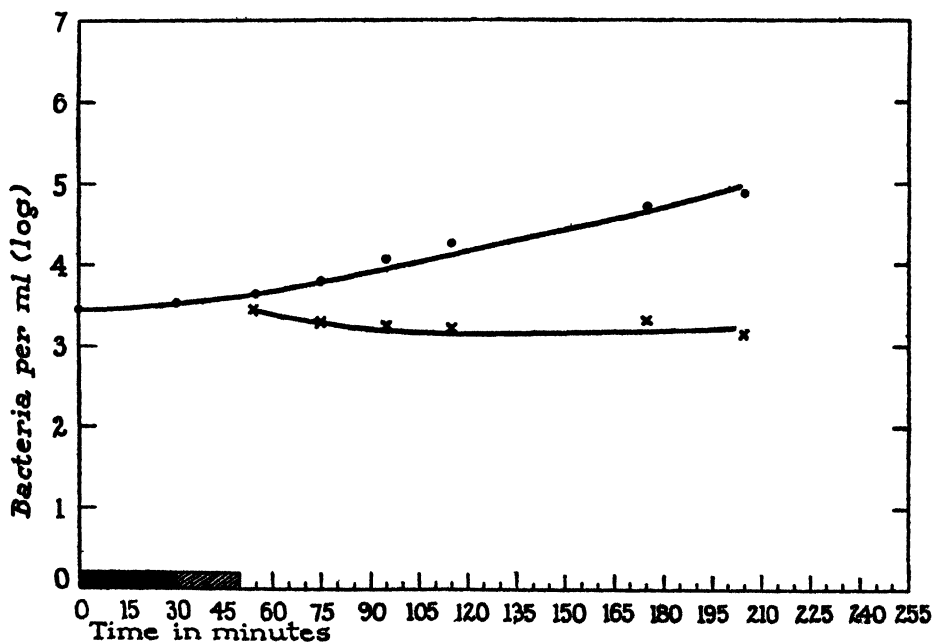


FIG. 5. EXPOSURE OF CULTURE TO 0.063 UNIT PER ML FOR 30 MINUTES

tion was continued. After the lapse of 30 minutes the penicillin-treated culture and an untreated control culture were centrifuged for 5 minutes. The supernatant was siphoned off, cells were immediately resuspended in warm broth,

centrifuged, resuspended in the original volume of warm broth, and returned to the incubator at 37 C. Samples were taken at intervals for counts of the viable cells. The results of a series of such experiments are presented in figures 2 to 5.

It will be noted that removal of growing cells from a culture medium by centrifugation and their transference to a fresh medium is accompanied by only a temporary reduction in the rate of reproduction. This is probably due to the reduction in temperature during the period of centrifugation at room temperature and the fact that during part of each run the packed cells were denied free access to the nutrients of the medium. This temporary effect on the rate of bacterial growth might have been predicted; however, the action of penicillin under these conditions was wholly unexpected. It will be observed that exposure of the growing culture to 1.0 unit of penicillin per ml led to a sharp drop in the number of viable cells, as in the first experiments. When, however, the cells were removed from a medium containing penicillin to one free of the drug, the decrease in the number of viable organisms continued for a time. Then the rate of death decreased until the viable cell count remained constant. This period was eventually followed by renewed growth, as has also been shown by other experiments. An explanation immediately suggested itself: the organisms present during this period of stationary population were the "persisters" described by Bigger (1944), organisms not in the proper physiologic state to be attacked by penicillin and therefore unaffected by it. Presumably, they were in a "resting," nonmultiplying stage. That this was not the whole explanation soon became apparent. When the period of exposure to penicillin was reduced to 15 minutes, the effect on the total number of viable cells was somewhat less, although there was a period of $3\frac{1}{2}$ hours before growth began. When the time of exposure was reduced to 5 minutes, essentially no bacterial deaths occurred. But for 3 hours after the removal of penicillin no bacterial multiplication occurred. Subsequent experiments (figure 5) showed that the effect could also be produced by a longer exposure to a lower concentration (0.06 units per ml) of penicillin. (Reference to figure 1 reveals that when a culture is continuously exposed to 0.06 units of penicillin per ml, the population does not begin to fall for about an hour after penicillin is added.) Very short exposure to the lower concentration of penicillin produced no significant effect.

DISCUSSION

Before any definite description of the mechanism of action of penicillin can be formulated, much more must be known than has been learned so far about the actual events which occur when bacteria are subjected to its action. As data become available, however, it is reasonable to examine currently held working hypotheses in the light of the new information. At the time of writing, the most widely accepted hypothesis as to the action of penicillin on bacteria holds that the substance acts on dividing cells only, and that cells escaping its action are either materially more resistant to penicillin than the average of the culture or are in an unsuitable physiologic state to be affected. With this hypothesis our experiments with relatively high concentrations of penicillin do not disagree. Destruction of each cell as it began or completed the process of division, or

death of a given percentage of the dividing cells, would produce the observed logarithmic death rate. As the concentration is reduced, however, a lag appears, and at the minimal inhibitory concentration it seems that a full generation is produced before bacterial death begins. Further, although the rate of growth may be lowered during this phase, it does not seem to be lowered significantly. When killing begins, it proceeds logarithmically for the period observed, although at a significantly lower rate than when the cells are exposed to a higher concentration of penicillin.

It might be presumed that the lag period of penicillin action during which there is no demonstrable effect on the bacteria is a period during which a critical concentration of the drug is being built up or the supply of some essential metabolite is being exhausted. On this basis, withdrawal of the drug before the lethal effect is apparent would be expected to allow prompt resumption of growth. In that event, however, a considerable period of time would be required for the bacteria to recover from the damage which had been done them, damage which was not yet evident at the time of removal of the penicillin. It may be important that there seems to be a time-concentration relationship in the production of this effect. Whether the interval between the removal of penicillin from the suspending medium and the resumption of growth is utilized by the bacteria in getting rid of the penicillin, repairing damaged enzyme systems, rebuilding stores of metabolites, or for some other purpose cannot be said at present.

CONCLUSIONS

At low penicillin concentrations, the lethal action of the antibiotic agent on *Staphylococcus* is preceded by a period of unaffected growth. At higher concentration the lethal action appears to begin without lag.

After removal of the bacterial cells to a penicillin-free medium, the effect of penicillin persists for a time. When *Staphylococcus* is exposed to 1 unit of penicillin per ml of culture medium, death of bacteria continues for a time after removal of the penicillin, and after a period of some 3 hours, growth is resumed. At lower concentrations of penicillin, exposure for a comparable time may lead to no deaths, but growth is inhibited for 3 hours after removal of the drug.

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ON THE PROCESS OF SPORULATION IN A STRAIN OF *BACILLUS CEREUS*

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Three modes of endospore formation are reported in the literature: (a) growth, from a single granule, (b) aggregation of granules, and (c) condensation of the protoplasm. (See Knaysi, 1944.)

Growth from a single granule was reported by a few of the early investigators for common organisms (*Bacillus subtilis*, *Bacillus anthracis*, *Bacillus megatherium*) which, later studies showed, sporulate by condensation of the protoplasm. With one or two exceptions, the same may be said of the aggregation of granules. The possibility that the difference between the three modes is due to a different state of the nuclear material was suggested by the author (1944, p. 99).

In the course of other studies, we had the opportunity to make careful observations on the mode of sporulation in strain C₃ of *Bacillus cereus*. The process can be readily observed in microcultures prepared as was described by the author (1940). Excellent results may be obtained by inoculating the droplet of agar with a spore suspension diluted with an equal volume of the medium: 100 ml of distilled water + 0.1 g of casein hydrolyzate + 0.1 g of K-phosphate mixture giving a pH of 6.8.

When lipoprotein inclusions are present, the first stage is the migration of these inclusions away from the locus where the endospore is to be formed (figures 1, 2, 12, 13). This takes place before delimitation of the forespore. It is probable that this migration takes place with the help of protoplasmic currents and is accompanied by considerable transport of protoplasmic material to the fertile part of the cell. This can be deduced from the fact that the latter can often be observed to increase in optical density while the region where the inclusions gather often seems to be largely transformed into a relatively huge vacuole (figures 2, 12, 13).

The second stage is the formation of a *forespore*. The first evidence of a forespore is the sudden appearance, in the hyaline, inclusion-free part of the sporangium, of a faint, elliptical contour which almost touches the cytoplasmic membrane of the sporangium (figures 1, 3, 4, 13). This contour line represents the intersection of a thin, ellipsoidal envelope with a plane containing the long axis of that envelope and normal to the axis of the microscope. This envelope consists of material similar in composition to that of the cytoplasmic membrane. It is of considerable interest that, in the bacterial cell, the separation of protoplasmic parts is always accomplished by films made out of this type of material; in cell division, these films are usually extensions of the cytoplasmic membrane; in the case of the forespore, it seems to appear *in situ*. At first, the protoplasm



FIG. 1. Portion of a 1-day-old microcolony, at 25 to 27 C, on the medium: 0.1 g of vitamin free casein hydrolyzate + 0.1 g of K-phosphate mixture (pH 6.8) + 1.5 g of bacto agar + 100 ml of distilled water. Note forespore in cell *a*.

FIG. 2. Same microcolony as in figure 1, 40 minutes later. Note completed endospore in cell *a* and the appearance of a forespore in cell *h*. Cells *c* and *f* are typical of cells about to sporulate.

within and without the envelope has the same refractive index (figures 3, 13), and the outline of the envelope is very difficult to see without staining; but the refractive index within the envelope rises rapidly, so that, usually within 10 minutes, the forespore becomes a highly refringent body (figures 3 to 5). There

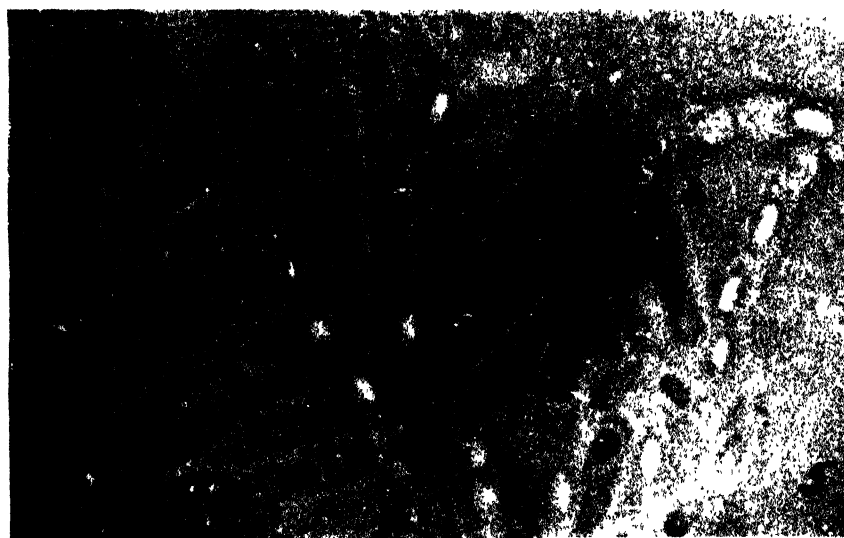
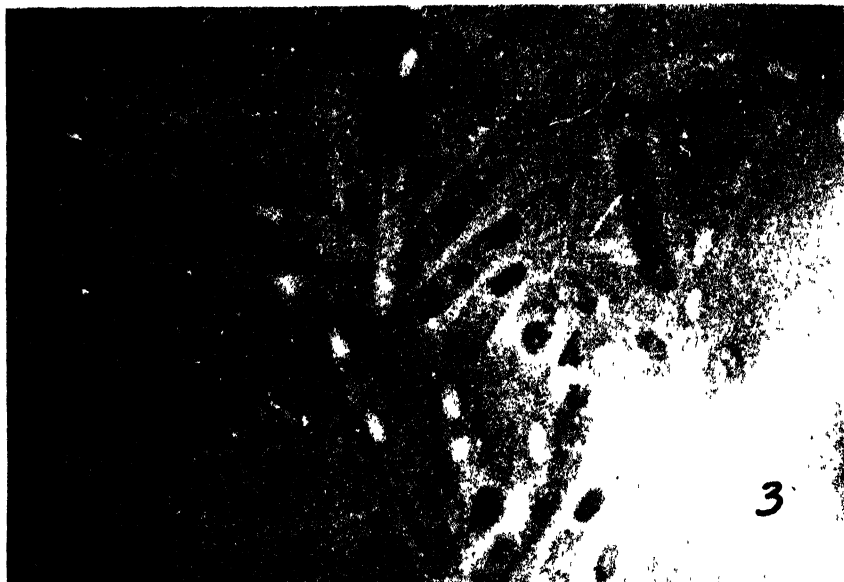


FIG. 3. Same microcolony as in figure 1, 1 hour and 20 minutes later. Note completed endospore in cell *h*, and the appearance of three forespores in cells *b* and *c*. Cell *f* also has sporulated.

FIG. 4. Same microcolony as in figure 1, 1 hour and 30 minutes later. Note an advanced stage of the forespore in cell *d*.

FIG. 5. Same microculture as in figure 1, 1 hour and 40 minutes later. Note completed endospores in cell *b*, *c*, and *d*, and the appearance of a forespore in cell *e*.

is not the slightest evidence for the contention of Meyer (1897, 1899, 1912) that the forespore contains a relatively huge vacuole. §

During a third stage, which may be called the stage of maturation, the forespore undergoes changes in form and size. It decreases in width with little or no change in length, thus assuming a more slender form and smaller volume (figures 1 to 6); it becomes the endospore. Measurements made on photomicrographic records of three developing endospores gave the data of table 1. These results are similar to those of Bayne-Jones and Petrilli (1933) for *Bacillus megatherium*.

This stage is followed by a period of two or more hours during which a sporangium, transferred to a new medium, remains unchanged; the inclusions remain intact and the endospore does not germinate (figures 16 to 18; see also Knaysi, 1945). This observation could be explained by postulating that the endospore must undergo further internal changes before it could germinate. Indeed, there is some basis for this assumption (Knaysi, 1946a). On the other hand, the fac-

TABLE 1
Morphological characteristics of forespore and endospore of strain C₁ of Bacillus cereus

No	a		b		F		I	
	forespore	endospore	forespore	endospore	forespore	endospore	forespore	endospore
	μ	μ	μ	μ			μ^1	μ^1
1	0.95	0.95	0.77	0.5	+0.344	+0.723	2.421	0.995
2	0.95	1.04	0.67	0.58	+0.500	+0.689	1.784	1.465
3	0.80	0.80	0.75	0.5	+0.116	+0.609	1.885	0.838
4	0.87	0.88	0.73	0.5	+0.291	+0.677	1.937	0.922

$$a = \frac{1}{2} \text{ of the length; } b = \frac{1}{3} \text{ of the width, } F = 1 - \frac{b^2}{a^2}; V = \frac{4}{3}\pi a b^2$$

that the sporangium and inclusions are preserved for a considerable period in a fresh medium indicates that the sporangium remains alive several hours after completion of the endospore, and that the endospore does not germinate until the sporangium dies of starvation; for the transfer of an old sporangium to a fresh medium hastens its disintegration or, if the sporangial wall is persistent, causes germination of the spore inside of the sporangium (Knaysi, 1938, p. 105). After this period of a few hours, the inclusions begin to disintegrate (cell c, figures 6 to 8), but the sporangium often breaks up before this disintegration is complete, and the inclusions or their residue is liberated into the medium (figure 9). In the strain investigated, the sporangium is usually persistent, in contrast with strain C₂ of *Bacillus mycoides* in which the sporangium disappears quickly after the stage of maturation.

It has been shown by the author (1946b) that, under certain conditions, endospores may be formed in inclusion-free sporangia. In such cases, there is no microscopic evidence of a preparatory stage, although transport of sub-microscopic protoplasmic material may be taking place. Visibly, the process



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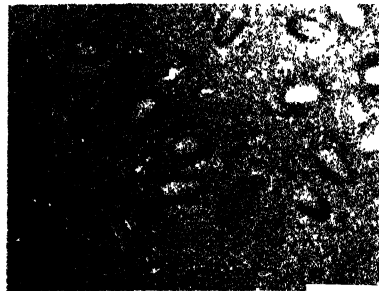


FIG. 6. Same microcolony as in figure 1, 2 hours later. Note completed endospore in cell *e* and the late stages of the forespore in cell *g*, note also the tendency to form a partition in cells *b* and *d*.

FIG. 7. Same microcolony as in figure 1, 4 hours and 12 minutes later. Note completed endospores in cell *g* and the tendency to form a partition in cells *e* and *g*; note also the two lipoprotein inclusions in cell *f*.

FIG. 8. Same microculture as in figure 1, 1 day and 45 minutes later. Note the disappearance of most of the lipoprotein inclusions, and the persistence and fusion of those in cell *f* into a larger granule.

FIG. 9. Same microculture as in figure 1, 3 days later. Note liberation of most residual lipoprotein inclusions as the sporangia disintegrate. It is possible that some of the scattered granules are fragments of the cytoplasmic membrane of the sporangium.

FIG. 10. Scale for figures 1 to 15

begins with the formation of the forespore in a hyaline cell. Subsequent changes are similar to those described above.

Position and orientation of the endospore. The position of the endospore may be anywhere in the sporangium and seems to be related to the morphology of the latter (figures 1 to 8, 11). The most frequent position is the subterminal, but strictly terminal spores are frequently seen (figure 11). In short sporangia, a central endospore may occasionally be seen (cell *i*, figure 6), and in long, slender sporangia, the terminal position becomes more frequent (cells *a* and *b*, figure 11).

The orientation of the endospore seems, at least partially, to depend on the difference between the width of the endospore and that of the sporangium. When this difference is small, the long axis of the endospore tends to coincide with that of the sporangium (figure 11). As the difference increases, however, the two axes tend to form an acute angle. Generally, this angle is small, but in a certain stubby type of sporangia it may reach 40 to 60 degrees. In other cases, shrinkage of the endospore during maturation results in a lateral translation (cells *j*, *k*, figure 6) so that the long axis of the spore and that of the sporangium are parallel. We have not observed any translational movement of the spore along the long axis of the cell as was reported by Lewis (1934) for *Bacillus mycoides*.

Form and size of the endospore. The general form of the endospore is ellipsoidal or, sometimes, oval; kidneylike spores also are occasionally seen. The ellipsoidal spores, however, vary in eccentricity from almost spherical ($E = +0.260$) to slender ($E = +0.779$), with a mean of about $E = +0.570$. The size of the endospore also varies considerably, and we observed midget spores which are not illustrated in this paper but will be the object of a future study. These midget spores may be found, lying obliquely, in sporangia of normal dimensions and appearance, but they seem to give at least a few generations of small, weak vegetative cells.

Table 2 contains the morphological characteristics of 10 endospores taken at random within a single microculture. The ratio of the volume of the spore to that of the sporangium varies between 0.11 and 0.14 (Bayne-Jones and Petrilli, 1933, reported for *Bacillus megatherium* the limits of 0.06 and 0.13). When a sporangium contains more than one endospore, the ratio of the total volume of the spores to that of the sporangium seems also to fall within those limits (table 2); this may be significant.

Number of endospores in a sporangium. The literature on the number of endospores per sporangium has been reviewed and discussed by the author (1944, pp. 100-102). The question is considerably clarified by the present study. Many sporangia contain only one spore each (cells *f* and *z* of figures 1 to 6). Equally frequent is the following phenomenon: a sporangium may contain a single, subterminal spore; subsequently, a plate, often of indefinite boundaries, may be formed at about the middle of that sporangium, and a second spore may be formed in the newly delimited half (cell *c*, figures 7 and 8; *j*, figures 3, 5, and 6); sometimes formation of both spores antedates that of the partition (cell *b*, figures 3, 5, and 6); usually, the plates ultimately split, showing that a disporic sporangium is potentially equivalent to two sporangia.

In filamentous growth (figure 11), two or more spores may be seen unseparated by partitions, although partitions may still be formed before disintegration of the filament.

TABLE 2
Morphological characteristics of a few endospores and sporangia of Bacillus cereus (strain C₈)

MICRO COLONY	ENDOSPORE				SPORANGIUM				$\frac{V_s}{V_{sp}}$
	<i>a</i>	<i>b</i>	<i>E</i>	<i>V</i>	<i>a</i>	<i>b</i>	<i>E</i>	<i>V</i>	
	μ	μ			μ	μ			
1	0.94	0.60	+0.590	1.405	2.39	1.00	+0.825	12.93	0.1085
	1.05	0.61	+0.664	1.635	3.00	0.94	+0.902	14.92	0.1095
	0.87	0.62	+0.496	1.400	2.20	1.05	+0.776	12.80	0.1095
	1.00	0.61	+0.628	1.558	2.32	0.92	+0.843	10.76	0.1447
	0.78	0.65	+0.311	1.380	2.30	0.91	+0.843	10.39	0.1328
2	1.05	0.63	+0.640	1.744	4.81	0.68	+0.980	13.29	0.1311
	1.05	0.56	+0.719	1.376					
	0.88	0.54	+0.628	1.075	13.50	0.55	+0.998	25.2	0.115*
	0.78	0.37	+0.779	0.448					
	0.70	0.44	+0.603	0.567		0.68			
	0.77	0.66	+0.260	1.403		0.71			

$a = \frac{1}{2}$ of the length, $b = \frac{1}{2}$ of the width, $E = 1 - \frac{b^2}{a^2}$, $V = \frac{4}{3}\pi a b^2$, V_s = volume of spore,

V_{sp} = volume of sporangium

* Endospores 2 to 4 of microcolony 2 were all in a single sporangium

TABLE 3
Changes in morphological characteristics of certain cells of Bacillus cereus (strain C₈) during sporulation

No.	<i>a</i>		<i>b</i>		<i>E</i>		<i>V</i>	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
	μ	μ	μ	μ			μ	μ^3
1	3.44	3.81	0.87	1.09	*	0.922	14.99	19.11
2	2.98	2.95	0.92	1.02	0.904	0.877	10.56	12.35
3	2.85	3.13	0.92	1.2	0.898	0.856	10.10	18.75

$a = \frac{1}{2}$ of the length, $b = \frac{1}{2}$ of the width, $E = 1 - \frac{b^2}{a^2}$, $V = \frac{4}{3}\pi a b^2$ except the initial state

of #1 (cylindrical) when $V = 2\pi b^2 (a - \frac{1}{3}b)$

* Cylindrical, the application of the formula is not theoretically correct but the formula gives the comparative value of +0.937.

We are unable to confirm the assertions of some early bacteriologists that a disporic sporangium is always twice as large as a monosporic one (compare cell *f* with *g* in figure 6). What determines the number of spores seems superficially to be not only the size of the sporangium but also the size of the spores. At the present stage, we do not wish to give our observations interpretations which might have a bearing on whether the endospore is a sexual or an asexual spore.

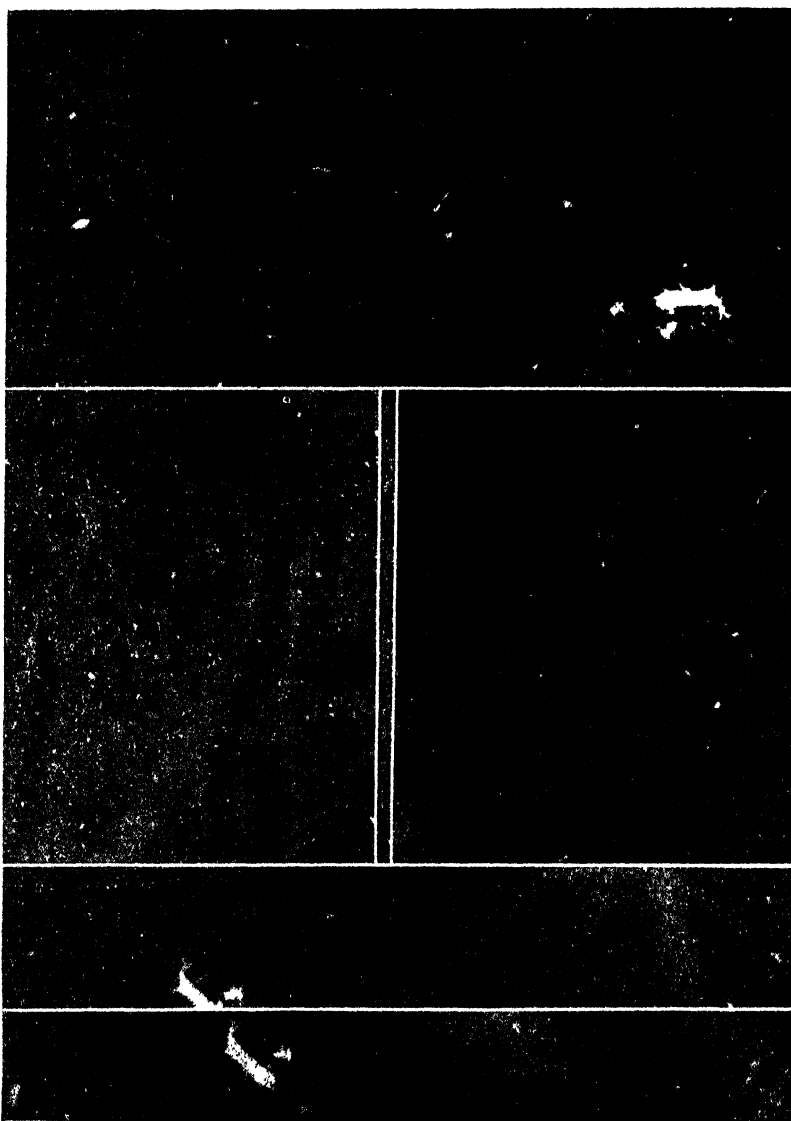


FIG. 11. Portion of a microculture as in figure 1. Filamentous cells showing two or more endospores per sporangium.

FIG. 12. Portion of a microculture similar to that of figure 1. At the end of one day it was disconnected and the cover glass with the adhering cells was placed on a droplet of the medium: 100 ml of meat infusion ($\frac{1}{2}$ strength) + 0.5 g of tryptone + 0.5 g of glucose + 1.5 g of bacto agar. The photograph was taken after an incubation for 52 minutes at 28 C. Note tendency of most cells to be ellipsoidal.

FIG. 13. The same portion of the microculture described in figure 12 after 4 hours and 38 minutes of incubation. Note the more definitely ellipsoidal form of cells *a*, *b*, and *c*. Cell *a* has already sporulated; cell *b* is preparing to sporulate; cell *c* has formed a forespore.

FIGS. 14 AND 15. The cells of figure 14 are at an early stage of preparation to form endospores; they are beginning to form and eliminate lipoprotein granules into the cytoplasm. As far as can be ascertained, they have developed into the sporangia of figure 15, 18 hours and 26 minutes later.

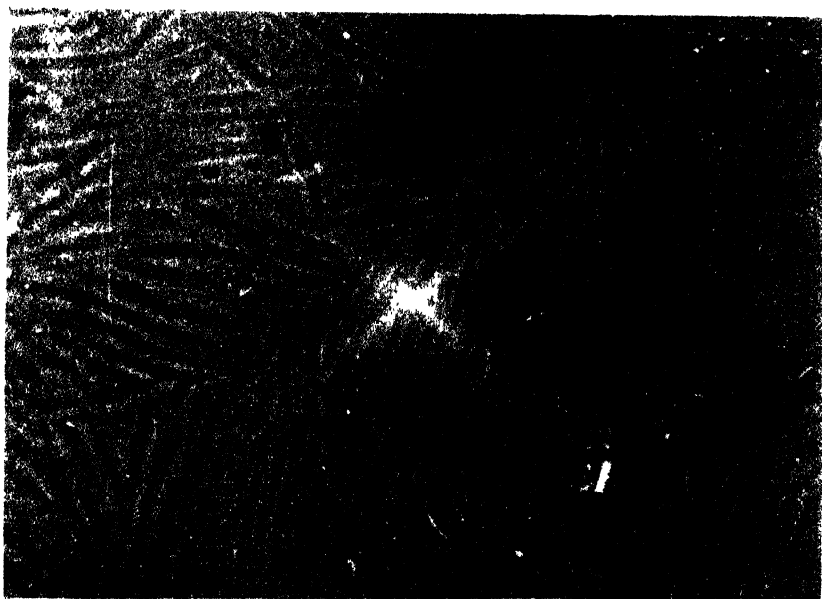


FIG. 16. A 19 hour old microculture similar to that of figure 1 was disconnected; the cells attached to the cover glass were mixed with a loopful of the medium: 100 ml of meat infusion ($\frac{1}{2}$ strength) + 0.5 g of tryptone + 0.5 g of glucose, and placed over a droplet of 1.5 per cent bacto agar, and so forth. A field containing 17 young sporangia and 37 granulated vegetative cells was picked for observation. Figure 16 is the appearance of that field after incubation of 3 hours and 37 minutes at 28 C. Note that none of the endospores has germinated.

FIG. 17. The major part of the field of figure 16 after 5 hours and 19 minutes. Note that none of the sporangia germinated, although growth of the vegetative cells has been considerable. The scale is the same as that of figure 16.

Changes in the form and size of the sporangium. In most cases there is no obvious change in the form or size of the cell during sporulation; occasionally, one observes around the forespore a constriction which may disappear during later stages. Not infrequently, however, one encounters cells which undergo definite changes in form and size during the period of migration of the inclusions. These changes may have begun shortly before. During that period, a cylindrical cell may swell laterally and assume an ellipsoidal form, or a cell which is already slightly ellipsoidal may decrease in excentricity. This swelling is not restricted to the region where the forespore later appears; it is often accompanied by growth in length (figures 12 and 13). The morphological characteristics of cells *a* to

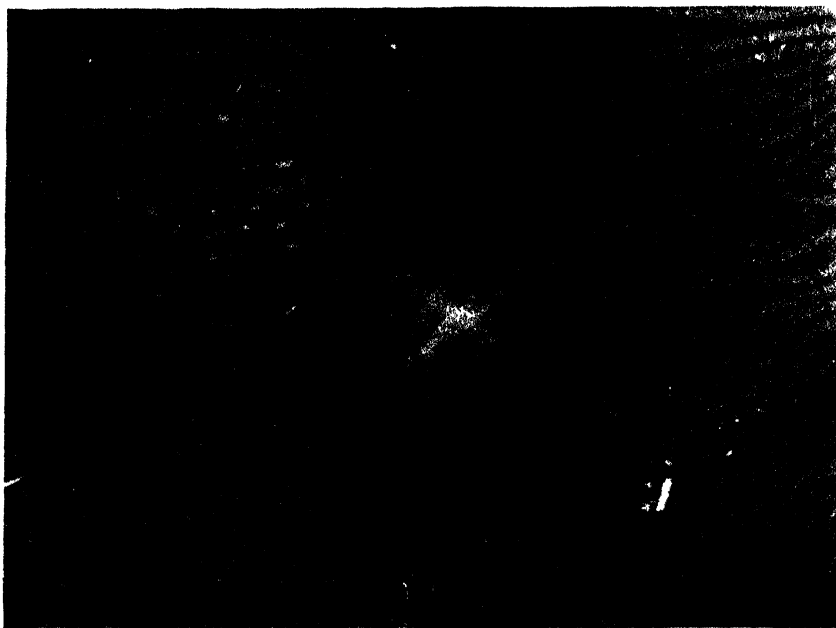


FIG. 18. The major part of the field of figure 16 after 6 hours and 39 minutes (See figure 17.) The scale is the same as that of figure 16.

c in their initial state (figure 12) and in their final state (figure 13) are given in table 3. Of the three cells, *b* was, initially, definitely cylindrical and its volume was calculated from the formula:

$$v = 2\pi b^2 (a - \frac{1}{3} b)$$

where *a* = half of the cell length (including the curved ends), and *b* = half of the width. This formula is much more convenient than the one previously given by the author (1944, p. 17):

$$v = 2\pi b^2 (a + \frac{2}{3} b)$$

in which *a* represented only the cylindrical part.

SUMMARY

Strain C₂ of *Bacillus cereus* forms endospores by condensation of the protoplasm. The cytological processes involved are described. When very young sporangia are transferred, together with vegetative cells, into a fresh medium, the spores do not germinate, and the sporangium and inclusions are preserved until the new culture reaches the sporulation stage.

The form and size of the endospore and its position in the sporangium are variable; its orientation seems to depend on the difference between its width and that of the sporangium; if the sporangium is much wider than the endospore, the latter tends to be oblique to the long axis of the sporangium.

The number of endospores in a sporangium is variable. There is a tendency, however, in sporangia containing more than one spore for them to be separated, often incompletely, into monosporous sporangia. More often, a large cell forms a spore; this is followed by the formation of a partition, and another spore is usually formed in the newly delimited half of the cell.

Only in a certain type of cells is sporulation preceded by enlargement of the sporangium. Sometimes midget endospores are formed.

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THE ACTION OF PENICILLIN ON SEVERAL GENERA OF THE ACTINOMYCETALES

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The sensitivity of the actinomycetes to penicillin has received little attention. The classification of the order advanced by Waksman and Henrici (1943) recognizes five genera (*Mycobacterium*, *Actinomyces*, *Nocardia*, *Streptomyces*, and *Micromonospora*) differing in morphological and physiological characteristics. Among the mycobacteria the resistance of the tubercle bacillus to penicillin has been known for some time, and a similar resistance has been shown for other members of the genus by Woodruff and Foster (1944). In this case the resistance is apparently due to the presence of an active penicillinase. Recent clinical reports (Hendrickson and Lehman, 1945; Dobson and Cutting, 1945) indicate that heavy dosages of penicillin are effective in some cases of actinomycosis caused by the anaerobic or microaerophilic organisms of the genus *Actinomyces*, and *in vitro* sensitivity of these organisms has been shown by Keeney *et al.* (1944). Unpublished observations of my own also indicate an *in vitro* sensitivity for these organisms. However, the problem of strain sensitivity in this genus warrants further investigation. Benedict and Iverson (1944) reported unsuccessful penicillin treatment of an eye infection caused by a species of *Nocardia*, although the organism showed *in vitro* sensitivity to 1 μ per ml concentration of penicillin. This seems to be the only report on the sensitivity of species of *Nocardia* to penicillin. Two strains of *Streptomyces* were tested by Waksman and Woodruff (1942) and found to be resistant to penicillin. Woodruff and Foster (1945) reported the presence of an active penicillinase in two species of the genus. Apparently no work has been done on the sensitivity of species of *Micromonospora*.

EXPERIMENTAL

A series of 17 strains of *Streptomyces*, 3 strains of *Nocardia gypsoides*, 5 strains of *Nocardia asteroides*, 1 strain of *Nocardia mexicana*, and 2 strains of *Micromonospora* were tested for sensitivity to penicillin by the method described below. The strains of *Streptomyces* were isolated from soil and were identified only as to genus. The strains of *Nocardia gypsoides* were obtained, respectively, from Dr. S. A. Waksman, Dr. C. W. Emmons, and from the collection of the late Dr. A. T. Henrici. The latter strain was a subculture of the type species. The 5 strains of *N. asteroides* consisted of 4 which have been previously studied for pathogenicity, etc. (Drake and Henrici, 1943) and a fifth received from Dr. D. S. Martin. The 2 strains of *Micromonospora* were received from Dr. Waksman and from Dr. W. W. Umbreit.

The organisms were grown on nutrient agar at 35 C for five transfers, then inoculated into 13 ml of melted nutrient agar and poured into petri plates. As soon as the plates had hardened, filter paper discs (9 mm in diameter) soaked in penicillin solutions were placed on the surface of the agar and the plates incubated at 35 C. A series of control plates inoculated with 15,000 spores of *Bacillus subtilis* per ml and with similar discs of penicillin-impregnated filter paper were prepared and incubated under the same conditions. Due to the mixed mycelial and bacillary growth of the *Nocardia*, and the presence of spores in the other two genera, no standard inocula could be devised for these organisms.

The faster and more convenient filter paper discs have been used by the author

TABLE 1
Action of penicillin on Streptomyces

STRAIN NO.	24 HOURS PENICILLIN (μ /ML)		48 HOURS PENICILLIN (μ /ML)	
	100	1,000	100	1,000
1	0	0	0	0
2	0	0	0	0
3	0	+p	0	0
4	—	—	11p	27
5	0	18	0	16
6	0	12p	0	12p
7	11p	17	11p	15p
8	0	0	0	0
9	0	11.5p	0	9p
10	11p	22.5	0	20p
11	0	+p	0	0
12	0	0	0	0
13	0	19	0	18
14	0	10p	0	0
15	0	10p	0	0
16	0	12p	0	0
17	12p	15p	0	15p

Figures indicate diameter of zone in mm, p indicates partial growth, 0 indicates no zone (normal growth), — indicates insufficient growth to measure zones, + indicates a zone too small to measure. There was no inhibition in concentrations of 0.1, 1.0, or 10 μ per ml.

for two years and the accuracy is comparable to that obtained with the more common cylinders. Similar findings with this method have been reported by others (Sherwood *et al.*, 1944; Vincent and Vincent, 1944).

The penicillin used was the commercial product of Eli Lilly and Company, purchased locally. The drug was dissolved in sterile, cold phosphate buffer of pH 6.2, and appropriate dilutions were made with the same buffer. Preliminary experiments with crude penicillin prepared in the laboratory had indicated little sensitivity of the actinomycetes, so five different concentrations were tested on the same plate. The control plates of *Bacillus subtilis* were run in quadruplicate for each set of dilutions, and the two higher concentrations of

penicillin were tested on different sets of plates than the lower concentrations. The dilutions used contained 0.1, 1.0, 10, 100, and 1000 μ per ml except for one organism (*Nocardia mexicana*) in which the dilution containing 0.1 μ per ml was omitted and a dilution containing 10,000 μ per ml was substituted.

Readings were made at the end of 24 and again at the end of 48 hours, since in some cases growth was too slight in the shorter interval. This also brought out the difference between temporary inhibition (by slowing of growth) and complete inhibition. This first phenomenon was shown by several organisms. The results are given in tables 1, 2, and 3. The results obtained with *Bacillus*

TABLE 2
Action of penicillin on Nocardia

ORGANISM	24 HOURS PENICILLIN (μ /ML)			48 HOURS PENICILLIN (μ /ML)		
	100	1,000	10,000	100	1,000	10,000
<i>N. asteroides</i>	0	0		0	0	
<i>N. asteroides</i>	0	0		0	0	
<i>N. asteroides</i>	0	0		0	0	
<i>N. asteroides</i> ..	0	10p		0	0	
<i>N. asteroides</i> (Martin).....	0	0		0	0	
<i>N. gypsoides</i> (Henrici).....	0	0		0	0	
<i>N. gypsoides</i> (Waksman) ..	10p	24		+p	24p	
<i>N. gypsoides</i> (Emmons)	0	0		0	0	
<i>N. mexicana</i> (Martin). .	0	0	16p	0	0	16p

See the explanatory note to table 1.

TABLE 3
Action of penicillin on Micromonospora

STRAIN	24 HOURS PENICILLIN (μ /ML)			48 HOURS PENICILLIN (μ /ML)		
	10	100	1,000	10	100	1,000
1. (Umbreit).	+p	14p	29	0	11p	30p
2. (Waksman).	—	—	—	0	24p	43p

See the explanatory note to table 1.

subtilis were in line with expectations and are not tabulated. There was marked inhibition of this organism by all concentrations of penicillin from 1.0 μ per ml up.

DISCUSSION

Among the 17 strains of *Streptomyces* only 3 showed any inhibition by 100 μ per ml, and in 1 of these this was overcome in 48 hours. In all 3 the inhibition was only partial and the zone consisted of decreased, but not completely suppressed, growth. At a concentration of 1,000 μ per ml of penicillin, 12 strains showed partial or complete inhibition in 24 hours, but only 8 were so affected after 48 hours. Of these 8, the inhibition was complete in 3 and partial in 5 others.

Only 1 of the 5 strains of *Nocardia asteroides* showed any inhibition, and in this case there was only a partial inhibition in 24 hours by a concentration of 1,000 μ per ml of penicillin, and that was completely overcome in 48 hours. One of the 3 strains of *N. gypsoides* showed a partial inhibition by 100 μ per ml and complete inhibition by 1,000 μ per ml in 24 hours, but after 48 hours there was only partial inhibition by these concentrations of penicillin. The single strain of *N. mexicana* showed a partial inhibition by 10,000 μ per ml but no effect from lower concentrations of penicillin.

The 2 strains of *Micromonospora* were slightly more sensitive since in the 1 showing sufficient growth for observation in 24 hours there was partial inhibition of a slight degree by 10 μ per ml and complete inhibition by 1,000 μ per ml. However, in 48 hours both strains showed only partial inhibition by the two highest concentrations of penicillin.

Preliminary experiments have shown the presence of potent penicillinase in liquid cultures of *Streptomyces* and *Nocardia*. A few observations on preparations from strains of the latter genus have shown that the penicillinase is much more heat-stable than has been reported for similar preparations from other organisms. A single experiment showed the antipenicillin activity was not completely destroyed by 30 minutes' exposure in a boiling water bath, although the activity was considerably reduced.

The lack of sensitivity shown by pathogenic species of *Nocardia* has some clinical importance. *In vitro* tests show that the concentration of penicillin required for inhibition is far beyond the concentration attainable in the body. In all cases of actinomycosis sufficient laboratory work should be done to identify the causative organism and to indicate its degree of sensitivity to penicillin. The favorable clinical reports have apparently all been in infections with the anaerobic *Actinomyces* whereas none have been reported as benefited by penicillin when the etiological agent was a species of *Nocardia*.

SUMMARY AND CONCLUSIONS

Seventeen strains of *Streptomyces* were tested for sensitivity to penicillin by the filter paper disc method. Eight strains were inhibited by concentrations of 1,000 μ per ml and of these only three were completely inhibited. Two strains showed partial inhibition by 100 μ per ml.

Five strains of *Nocardia asteroides* were tested in the same manner. Only one strain showed any inhibition at penicillin concentrations of 1,000 μ per ml or less, and this partial inhibition was overcome in 48 hours.

One of three strains of *Nocardia gypsoides* showed partial inhibition by 100 and 1,000 μ per ml, whereas the others were resistant to these concentrations of penicillin.

One strain of *Nocardia mexicana* was resistant to 1,000 μ per ml but was partially inhibited by 10,000 μ per ml concentration of penicillin.

Two strains of *Micromonospora* were partially inhibited by 100 and 1,000 μ per ml of penicillin but not by lower concentrations.

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A METHOD FOR RESTORING AND MAINTAINING THE PHENOL RESISTANCE OF CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS

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Despite the many acknowledged limitations of the phenol coefficient test, the fact remains that it is still the most generally useful and practical test that has thus far been devised for estimating the relative germicidal activity of phenolic compounds. The modification of this test usually employed in this country is that described by Ruehle and Brewer (1931) and commonly accepted as the Food and Drug Administration method. If the directions described by these investigators in Circular no. 198 are carefully followed, workers in different laboratories, testing the same disinfectant, are usually able to obtain similar results—one of the salient features of this test. However, there is one variable in the test, namely the phenol resistance of the test organism, which at times appears uncontrollable. Ruehle and Brewer set certain minimum limits of phenol resistance for the two test organisms, *Eberthella typhosa* (Hopkins strain) and *Staphylococcus aureus*; and although it is generally agreed that these limits are easily maintained for *E. typhosa*, the *S. aureus* strains are apparently subject to unpredictable fluctuations in phenol resistance despite rigid adherence to methods of culture and transfer prescribed in Circular no. 198. This factor has caused justifiable consternation in those who rely upon this organism for practical day-to-day control testing of antiseptics and germicides, since it often means the loss of an entire day's work when the test organism is subsequently found to exhibit substandard phenol resistance.

The cause of these fluctuations in resistance is not clearly understood at the present time. Undoubtedly different brands or even batches of peptone may alter the resistance of the organisms as Brewer (1943) found in the case of *E. typhosa*. Recently Wolf (1945) has prepared a medium which maintains the resistance of *S. aureus* more uniformly than the standard F.D.A. broth. At the present time, however, attempts to stabilize the phenol resistance of *S. aureus* by variations in the medium can only be made on an empirical basis, since the relationship between the metabolism of the organism and its phenol resistance is not known. In view of this situation, it is believed that the present report, also based on an empirical finding, may be of assistance to those who occasionally experience difficulty in restoring and maintaining the standard phenol resistance of *S. aureus*.

After preliminary experiments with different brands of peptone and meat extract that yielded unsatisfactory results in attempting to restore the phenol resistance of *S. aureus*, it was found that the incubation of certain strains at 40 C restored and maintained the desired resistance. Since this appeared to be a

possible solution for obtaining satisfactory cultures for phenol coefficient determinations, a series of experiments, outlined below, was conducted to determine the practicability of such a procedure.

EXPERIMENTAL

The directions described in Circular no. 198 were followed in performing all phenol coefficient tests. Armour's peptone (batch C2806) and beef extract

TABLE 1
Phenol resistance of S. aureus grown at 37 C and 40 C

CULTURE NO.	TRANSFER NO.	CULTURE INCUBATED AT	TESTS CONDUCTED AT 20 C						TESTS CONDUCTED AT 37 C					
			Phenol dilution						Phenol dilution					
			1:60			1:70			1:80			1:90		
			Minutes exposed to phenol						Minutes exposed to phenol					
			5	10	15	5	10	15	5	10	15	5	10	15
209-2	4	37 C	-	-	-	+	+	-						
		40 C	+	-	-	+	+	+						
	6	37 C	-	-	-	+	+	-	-	-	-	+	-	-
		40 C	+	-	-	+	+	+	-	-	-	+	+	+
	12	37 C	-	-	-	+	+	-	-	-	-	+	-	-
		40 C	+	-	-	+	+	-	+	-	-	+	+	+
209-1	4	37 C	-	-	-	-	-	-						
		40 C	+	-	-	+	+	+						
	6	37 C	-	-	-	+	-	-	-	-	-	-	-	-
		40 C	+	-	-	+	+	+	-	-	-	+	+	-
	12	37 C	-	-	-	+	-	-	-	-	-	-	-	-
		40 C	+	-	-	+	+	+	+	-	-	+	+	+
209-B	4	37 C	+	-	-	+	+	+						
		40 C	+	-	-	+	+	+						
	6	37 C	-	-	-	+	-	-	-	-	-	+	-	-
		40 C	-	-	-	+	-	-	-	-	-	+	-	-
	11	37 C	-	-	-	+	-	-	-	-	-	+	-	-
		40 C	+	-	-	+	+	+	-	-	-	+	+	+

+ = Growth in subculture tube after 48 hours' incubation at 37 C.

- = No growth in subculture tube after 48 hours' incubation at 37 C.

(batch 12414) were employed in the preparation of all media. A Friez recording thermometer and hygrometer were used in both the 37 C and 40 C incubators. Variations between 20 per cent and 60 per cent relative humidity were tested in the incubators, but these did not appear to affect the phenol resistance of the test organisms.

In the first series of experiments, four cultures of *S. aureus* (strain 209) from different sources were transferred daily, one set of strains being incubated at 37 C and the other at 40 C. After a varying number of transfers, the cultures were tested for their phenol resistance both at 20 C and at 37 C, since Slocum

(1945) found that frequently *S. aureus* had standard resistance when tested at 20 C but showed substandard resistance at 37 C. These experiments were repeated 40 times over a period of 4 months, and results similar to those shown in table 1 were obtained. Here three of the strains were tested under the conditions indicated in the table.

TABLE 2
Phenol resistance of S. aureus grown at 37 C and 40 C

CULTURE NO.	TRANSFER NO.	CULTURE INCUBATED AT	TESTS CONDUCTED AT 20 C					
			Phenol dilution					
			1:60			1:70		
			Minutes exposed to phenol					
			5	10	15	5	10	15
N. E.	4	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	6	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	11	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	16	37 C	—	—	—	+	—	—
		40 C	—	—	—	—	—	—
S. T.	4	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	7	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	+	—
	11	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	14	37 C	—	—	—	—	—	—
		40 C	—	—	—	+	—	—
16	37 C	—	—	—	+	—	—	
	40 C	—	—	—	+	—	—	
W. P.	4	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	+	—
	6	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—
	11	37 C	—	—	—	+	—	—
		40 C	—	—	—	+	—	—

+ = Growth in subculture tubes after 48 hours' incubation at 37 C.

— = No growth in subculture tubes after 48 hours' incubation at 37 C.

Table 1 shows that with but one exception (strain 209-B, fourth transfer) these strains exhibited substandard phenol resistance when grown at 37 C but responded to 40 C incubation with the required resistance. In some cases there was a slight loss in resistance when the tests were conducted at 37 C, thus confirming Slocum's (1945) experience. The increased resistance obtained by culturing at 40 C is only transient, for even after strains have maintained stand-

ard resistance for 16 transfers at 40 C, when they are again incubated at 37 C they show substandard resistance after the first or second transfer. This fact, however, does not decrease the practical value of incubation at an elevated temperature, for cultures have maintained standard resistance at 40 C for 23 consecutive transfers without any indication of granularity or roughness developing. The strain 209 cultures that were initially substandard or that developed substandard resistance when incubated at 37 C usually exhibited standard resistance after the first or second transfer incubated at 40 C.

Although incubation at 40 C has consistently increased the resistance of each of the 209 strains tested, there appear to be many strains of *S. aureus* which do not respond to this stimulus. In table 2 are shown the results of testing three of these strains which, with two exceptions, failed to show any increase in resistance

TABLE 3
Mercuric chloride resistance of S. aureus grown at 37 C and 40 C

CULTURE NO.	TRANSFER NO.	CULTURE INCUBATED AT	TESTS CONDUCTED AT 20 C								
			Mercuric chloride dilution								
			1:1,000			1:2,000			1:3,000		
			Minutes exposed to mercuric chloride								
			5	10	15	5	10	15	5	10	15
209-1	5	37 C	-	-	-	-	-	-	-	-	-
		40 C	-	-	-	+	+	+	+	+	+
	19	37 C	-	-	-	-	-	-	+	-	-
		40 C	-	-	-	+	-	-	+	+	+
209-2	5	37 C	-	-	-	+	-	-	+	+	+
		40 C	-	-	-	+	+	-	+	+	+
	19	37 C	-	-	-	-	-	-	-	-	-
		40 C	-	-	-	+	+	-	+	+	+

+ = Growth in subculture tube after 48 hours' incubation at 37 C.

- = No growth in subculture tube after 48 hours' incubation at 37 C.

when incubated at 40 C. Seven additional strains of *S. aureus*, recently isolated from pathological material, also failed to respond to elevated incubation temperatures.

Apparently incubation at 40 C does not enhance phenol resistance, even after a prolonged series of transfers, unless the organisms possess some inherent potential quality of resistance, similar to that of the 209 strain. The failure of many strains of *S. aureus* to respond to an elevated temperature is not a real limitation to this procedure, for most workers using *S. aureus* for phenol coefficient tests now employ the 209 strain, and on the basis of the results observed in this study, cultures of the 209 strain are generally amenable to enhancement of phenol resistance by incubation at 40 C.

Although the phenol coefficient test is not applicable to antiseptics other than phenolic compounds, for the reasons stated in Circular no. 198, it is frequently

useful in testing newly developed nonphenolic antiseptics to compare their activity with that of phenol. It was therefore believed of interest to determine the effect of incubating cultures of the 209 strain at 40 C on their resistance to another type of antiseptic. In table 3 the resistance of two strains against mercuric chloride is shown.

It will be observed that both strains, when incubated at 40 C, show an increase in resistance to mercuric chloride. Apparently the increased phenol resistance induced by incubation at 40 C may also be exhibited toward other types of antiseptics.

DISCUSSION

The demonstration of increased resistance of *S. aureus* to phenol when incubated above 37 C is not new, for Chick (1910) described it and more recently Smyth (1934) made a similar finding. So far as the writers know, however, the practical application of this phenomenon in conducting phenol coefficient tests has not been previously explored. The use of 40 C cultures has frequently been helpful in our laboratory at certain times of the year when the 37 C cultures were persistently substandard for a number of days. It is believed that this procedure may also be of use to others who have experienced similar difficulties from time to time.

A few strains were incubated at 45 C, but this temperature did not raise the resistance of the cultures of strain 209 beyond that achieved by incubation at 40 C, nor did it increase the resistance of the other strains that failed to respond at all to incubation at 40 C.

Although incubation of *S. aureus* at 40 C is at variance with the procedures outlined in Circular no. 198, the use of such cultures would appear justifiable for phenol coefficient determinations so long as they maintain the standard phenol resistance. Moreover, when phenol coefficient tests are used on nonphenolic antiseptics for experimental purposes, the use of 40 C cultures should also be satisfactory. It is hoped that incubation of the test cultures at 40 C will prove helpful in improving the practicability of the phenol coefficient test, just as a change in the standard medium, suggested by Wolf (1945), seeks the same objective.

SUMMARY

Incubation of four cultures of *Staphylococcus aureus* (strain 209) at 40 C usually restored and maintained their phenol resistance at the standard prescribed by the F.D.A. for phenol coefficient determinations.

Phenol resistance increased by incubation at 40 C was maintained when the phenol coefficient test was conducted at 20 C and only slightly diminished when tests were conducted at 37 C.

Standard phenol resistance, maintained by incubation at 40 C, was rapidly lost when the cultures were subsequently incubated at 37 C.

When strains of *S. aureus*, other than 209, were repeatedly transferred and incubated at 40 C, they failed to exhibit increased phenol resistance.

Incubation of certain *S. aureus* cultures at 40 C to restore or maintain standard phenol resistance is recommended when incubation at 37 C fails to produce the required resistance.

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FURTHER NOTES ON VARIATION IN CERTAIN SAPROPHYTIC ACTINOMYCETES¹

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Variation in certain saprophytic soil actinomycetes has been reported (Jones, 1940). Since then observations have accumulated on the effect of the amount of inoculum, prolonged culture in soil compared with broth or agar media, and the selection of temporary variants.

THE EFFECT OF THE AMOUNT OF INOCULUM

The effect of the amount of inoculum was tested on the most variable characteristics of the writer's cultures: (1) pigmentation and (2) digestion of calcium malate.

The effect on pigmentation was observed in organism 47-13 secured by the writer from a forest soil and established in culture from a single spore in 1938. On agar media this organism loses its ability to form aerial hyphae and spores after a few transplants. The colonies, on ordinary dilution plates of glycerol nitrate agar, are all colorless until about the sixth day when some begin to appear greenish black, brown, or irregularly variegated. The color increases at an unequal rate in different colonies, and within a colony. A few remain colorless, particularly the smaller ones. The writer (1940) classified these variations as continuous or fluctuating, but stated, "The pigment develops in a few of the larger colonies perhaps because they are innately more active, or they may have arisen from a larger fragment of the inoculum in a very fortunate position within the agar with reference to oxygen supply."

It has since been found that a heavy suspension from cultures recently grown in sterilized soil gives entirely, or almost entirely, pigmented colonies; e.g., this is true of a plate inoculated at 25 successive points with a needle dipped only once in the suspension. However, if the inoculum is carried over to three plates (75 inoculations), or if the suspension is diluted, differences in color occur with the faintly pigmented or colorless colonies, especially at the end of the series (fig. 1, 1). A typical run of 17 replicates averaged 95 per cent colored colonies on plate 1, 86 per cent on plate 2, and 73 per cent on plate 3. After several subcultures, pigmentation decreases and there may be no correlation between it and the amount of inoculum (fig. 1, 2), e.g., 10 replicates averaged 9 per cent pigmented colonies on plate 1, 22 per cent on plate 2, and 20 per cent on plate 3, after being subcultured on glycerol nitrate agar for a year. Colorless colonies ordinarily yield some colored derivatives. The pigmentation of these is unaffected by the inoculum, regardless of the length of time on laboratory media. In one trial, 122 colorless colonies were used for stab transfers with the following

¹Paper number 829 from the Department of Botany of the University of Michigan.

results: 10 produced no growth, 28 formed only colorless colonies (fig. 1, 3), 76 formed colored and colorless colonies, without reference to the quantity of inoculum, and 8 yielded entirely colored colonies.

The amount of inoculum affects the digestion of calcium malate in organism 21-2 which was secured by the writer from a grassland soil and established from a single spore in 1939. The growth is a compact vegetative stroma, which develops white aerial hyphae and conidia on Krainsky's calcium malate agar. Practically every plate prepared from a suspension of the macerated stroma into melted

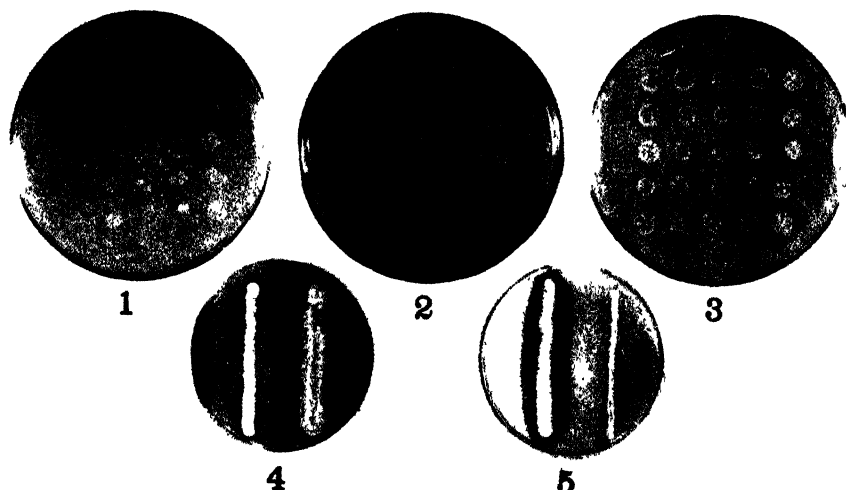


FIGURE 1

Nos. 1, 2, and 3. Organism 47-13 on glycerol nitrate agar.

No. 1. Growth freshly transplanted from soil culture. Pigmentation correlated with amount of inoculum.

No. 2. Growth from a culture grown for a year on laboratory media. No correlation between pigmentation and amount of inoculum.

No. 3. Colorless form developed by selection of colorless variants.

No. 4. Organism 21-3 on starch agar treated with Lugol's iodine solution. Left streak from soil culture shows digestion of starch. Right streak from broth culture shows slight digestion of starch.

No. 5. Organism 63-7 on calcium malate agar. Left streak from glycerol nutrient agar shows digestion of calcium malate. Right streak from soil shows no digestion.

agar shows variation in the rate in which colonies digest calcium malate. The most active, and usually the largest, colonies produce clear zones in the medium in 3 days, whereas others may take as long as 21 days. Except for this characteristic, 21-2 has been a very stable organism.

The effect of the amount of the inoculum was tested 32 times using cultures which had grown from 1 to 13 months on calcium malate agar. There were 5 to 20 replicate plates prepared from 187 colonies. The inoculum was distributed to 25 successive points on a plate—5 rows of 5 inoculations. The percentage of colonies to digest calcium malate in row 1, which received the

heaviest inoculum, was always higher than row 5 which received the least, as the following data show:

<i>Trial</i>	<i>Row 1</i> %	<i>Row 5</i> %	<i>Trial</i>	<i>Row 1</i> %	<i>Row 5</i> %
1	100	36	17	37	25
2	100	70	18	71	33
3	63	54	19	46	33
4	38	4	20	43	8
5	90	51	21	54	11
6	59	29	22	71	21
7	81	31	23	45	2
8	69	14	24	62	8
9	86	66	25	58	20
10	6	3	26	95	51
11	38	25	27	79	27
12	34	2	28	86	50
13	17	0	29	80	34
14	25	0	30	53	5
15	27	0	31	80	4
16	40	0	32	80	27

A comparison of the actual percentages between the trials is pointless because of such variables as the concentrations of inocula and the history on artificial media. In 17 trials there was a row-by-row correlation, i.e., digestion was most rapid in row 1 and declined progressively in the remaining rows without exception. In 7 trials one row deviated, in 6 trials 2 rows deviated, and in 2 trials 3 rows were not in decreasing order.

PROLONGED CULTURE IN SOIL COMPARED WITH BROTH OR AGAR MEDIA

The characteristics of an actinomycete are so dependent on the substrate that it is necessary to use synthetic media in taxonomic studies. Not much attention has been paid to the fact, however, that cultural characteristics on a given medium may be greatly affected by the substrate on which the organism previously grew. As an extreme example, the writer grew 5 actinomycetes and 4 of their mutants for 4 years on (1) moist, sterilized soil; (2) a broth containing 10 g glycerol, 5 g calcium malate, 5 g glucose, 2 g NaNO₃, 0.5 g KCl, 0.5 g MgSO₄, 1 g K₂HPO₄, and a trace of FeSO₄ in 1 liter of distilled water; and (3) nutrient agar fortified with 2 per cent glycerol. Some of the growths did not survive prolonged culture on laboratory media. The organisms were finally transferred to glycerol nutrient agar, subcultured once, and then grown on the synthetic media: Czapek's sucrose nitrate agar, Krainsky's glucose asparagine agar, Krainsky's calcium malate agar, and Waksman's starch agar.

Every organism showed important differences between the growths from soil and from laboratory media (fig. 1, 4 and 5) which were maintained over many transfers, until the test media themselves brought changes. Mutant 21-11c was least altered, showing only one deviation: on starch agar, the soil culture produced spores sparingly, whereas from broth the growth was asporous. The

parent organism, 21-11, of this mutant showed among the greatest contrasts; hence it can be used to illustrate the effects produced (see table 1).

The following is a list of all the characteristics which were altered by continuous culture on a particular substrate: (1) thickness, surface, margin, and texture of the mycelium as viewed macroscopically; (2) rate of growth; (3) presence or absence of aerial hyphae and conidia; (4) distribution of aerial hyphae and conidia; (5) deliquescence on the surface of the growth; (6) presence or absence of pigments; (7) type of color; and (8) rate of digestion of calcium malate and starch.

TABLE 1

Characteristics of organism 21-11 on synthetic media which depended on the previous substrate

SYNTHETIC MEDIUM	PREVIOUS SUBSTRATE	
	Soil	Agar
Waksman's starch	Weakly diastatic Spores drab Spores uniformly cover growth	Strongly diastatic Spores pink Spores in wedge-shaped sectors
Krainsky's glucose asparagine	Spores cinnamon-drab Spores uniformly cover growth	Spores Japanese-rose Spores in rectangular patches
Czapek's sucrose ni- trate	Spores cinnamon-drab Spores uniformly cover growth	Spores white Spores sparse
Krainsky's calcium malate	Rapid digestion of calcium malate Streak thin, smooth-edged White aerial hyphae	Slow digestion Streak of small colonies No aerial hyphae

The writer (1940) has stated, "In moist soil at room temperature, the strains may be maintained for months, perhaps years, without losing their original properties." Subsequent results confirm this and indicate the practicability of carrying stock cultures in sterilized soil. Cultures 4½ years in sterilized soil, without transfer, when plated on synthetic agar show the color, sporulation, and enzymatic behavior which characterized the original isolates from nature. A white-spored mutant of 21-11, which originated on laboratory media, gave 66 per cent colored-spored colonies in the first plating after 4½ years in soil; however, all subcultures were white-spored, irrespective of the colony used for inoculation.

THE SELECTION OF TEMPORARY VARIANTS

It has been reported (Jones, 1940): "An attempt was made to obtain a pure line of black pigmented colonies (of 47-13) by subculturing from the darkest colony in each 'generation.' The results were very irregular, although as high as 95 per cent pigmented colonies have been secured in a 50-plate transfer. The colorless segregates in this line gave 70 to 89 per cent pigmented colonies. Se-

lection for a colorless type was unsuccessful; a varying number of pigmented colonies always appeared and many of the colorless isolates were low in vitality." The variations were considered to be continuous or fluctuating, such as obtain for a characteristic within a pure line.

Subsequent studies on selection have been complicated by the gradual loss of pigmentation through prolonged culture on artificial media. For example, after 31 months on glycerol nitrate agar, 24 per cent of the colorless colonies tested produced only colorless growths, and many colored colonies yielded less than 10 per cent of pigmented derivatives. The color was restored after the colorless growths were cultured for 2 months in sterilized soil, or sometimes after growing on Krainsky's glucose asparagine or glycerol nutrient agar. However, the restoration was only partial in 11 out of 28 instances, for the color was either unusually weak or very few colonies developed color. Pure, permanently colorless growths would probably arise eventually as a direct effect of the medium. The selection of colorless colonies for each subculture would accelerate the change.

Variations in color in organism 47-13 can no longer be regarded as fluctuating, in the strict sense, since the frequency of types can be altered under certain conditions by the quantity of inoculum and by artificial selection. It must be stressed, however, that individual cultures may deviate widely from the usual trends due to obscure physiological conditions. The frequently observed, variegated colonies indicate that sharp and considerable differences may occur in the structurally undifferentiated mycelium.

The selection of colonies unable to digest calcium malate in organism 21-2 did not in itself lead to an inactive line. Transfers were made from soil to glycerol nutrient agar, and after one transfer, platings were begun on calcium malate agar. Each plate was inoculated at 25 successive points, giving diminishing amounts of inocula. In the first plating, 81 per cent of the colonies developed; at 6 days 94 per cent of these formed clear zones in the medium, and at 8 days all colonies had cleared it. Twenty-five successive subcultures were made from colonies which failed to show macroscopic evidence of digestion in 6 days. The percentage of colonies to digest the medium in 6 days in the first 9 subcultures was very irregular, as follows: 30, 98, 44, 20, 74, 74, 87, 68, and 83. Beginning with the tenth transfer, the rate of digestion decreased, but since this was likewise true in platings from active colonies it was a direct effect of the medium and not of selection. Activity was restored by culturing in sterilized soil. In the twenty-fifth transfer, 42 per cent of the colonies developed; only 9 per cent showed digestion in 10 days, and some colonies took up to 45 days to form clear zones in the medium.

DISCUSSION

Some actinomycetes, established in culture from single cells, show a wide range of temporary variations for a given characteristic. When freshly isolated, the entire range of types is secured irrespective of the colony chosen as an inoculum. Stanier (1942), for example, found that this was true of his PG strains of *Actinomyces coelicolor*, which gave a 95 to 5 ratio of apparently non-agar-decomposing

to agar-decomposing colonies. He believed it "unavoidable that the process of conidium formation was in some way responsible for the observed phenomena," and favored the cytological findings of Badian (1936) as an explanation. Whatever may be the cause of the color variations in the writer's organism 47-13, it cannot be this, as the growths were entirely of substrate mycelium. In organism 21-2, in which colonies varied in their rate of calcium malate digestion, there was a sparse production of conidia.

Stanier's PG strains increased in activity with subculturing and finally showed no differences in the rate at which colonies digested the agar. The writer's cultures behaved in just the opposite fashion: calcium malate digestion and pigmentation (which were affected by the amount of inoculum) declined after prolonged subculturing on the requisite medium. The selection of colorless variants speeded the loss of pigmentation. The characteristics were restored, at least partially, by culturing the organisms (which were not necessarily otherwise debilitated) in sterilized soil or on a rich nutrient agar. The characteristics of an actinomycete on a test medium may depend upon the previous substrate.

Sterilized soil not only restores characteristics lost on synthetic media but also serves excellently for stock cultures. The writer has such cultures, kept 4½ years at room temperature without transfer, which have the original characteristics on synthetic media of the organism freshly isolated from natural soil. Only one permanent variant has developed in the soil cultures, a fact which indicates that the rate of production of permanent variants in nature must be low for these strains. Schaal (1944) has amply demonstrated that sectoring in actinomycetes is a racial character. It may be that the writer's organisms are unusually stable. They have produced very few sectors on agar media; however, the colonies were not maintained for the long period of time which Schaal has found to be necessary.

SUMMARY

Certain saprophytic actinomycetes, established in culture from single cells, show a wide range of temporary variations on a synthetic medium. When freshly isolated, the entire range of types for a given characteristic is secured, irrespective of the colony chosen as an inoculum. The frequency of types may be affected by the amount of inoculum and by continued selection. Prolonged subculturing on laboratory media leads to a diminution or loss of certain characteristics. At least partial restoration results from culturing in sterilized soil, which serves as an excellent medium.

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A STUDY OF THE DISSOCIATIVE BEHAVIOR OF *PSEUDOMONAS AERUGINOSA*^{1,2}

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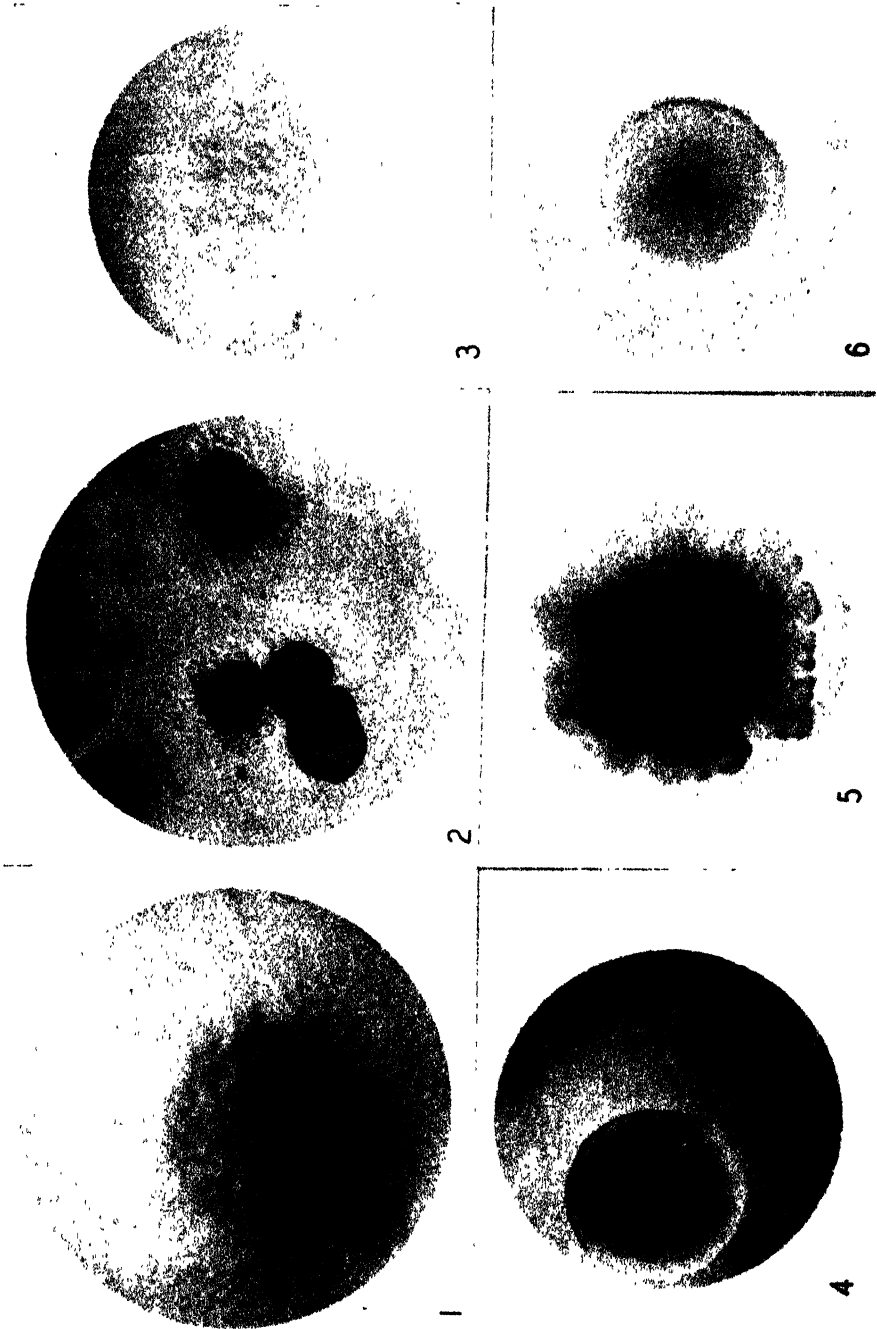
Since 1860, when Fordos published his experimental observations concerning the blue-green stain that sometimes appeared on surgical dressings, numerous articles dealing with the organisms commonly assigned to the species *Pseudomonas aeruginosa* have appeared in the literature. Because of its wide and varied range of activity, the definition of this species presents considerable difficulties, and there exists a need for an accurate method of identifying this microorganism. One organism has frequently been described under two names, and specific names have been given to organisms which appear to be merely types or varieties of the species.

These organisms are widely distributed in nature, existing usually as saprophytes, although occasionally giving rise to pathological lesions and generalized infection in man (Lartigau, 1898; Waite, 1908; Fraenkel, 1917; Pons, 1927). Scattered throughout the literature are numerous reports dealing with their pathogenic potentialities for plants (Paine and Branfoot, 1924; Harris, 1940; Nagbski, 1941; Elrod and Braun, 1941, 1942; Reid *et al.*, 1942). Perhaps the most outstanding feature of *P. aeruginosa* is the ability to produce a blue-green, chloroform-soluble pigment, pyocyanin. Attempts to determine the nature of the pigment and the circumstances under which the chromogenic properties are manifested have resulted in considerable confusion. Some investigators believe that only one pigment is formed by the different variants and that these variants differ chiefly in their ability to produce ammonia; on the other hand, some maintain that one strain is simultaneously able to produce as many as four distinct pigments. Between these two extremes, we find those who claim that different metabolic pigments may be formed by varying the nutrients of the culture medium.

Jordan (1899) separated the species of *Pseudomonas aeruginosa* into four varieties: (1) pyocyanigenic and fluorescogenic, (2) pyocyanigenic only, (3) fluorescogenic only, and (4) nonchromogenic. In later experiments, Jordan (1903) concluded, from the study of 58 strains of these organisms, that the ability to liquefy gelatin was closely related with the ability to coagulate milk. The organisms capable of liquefying gelatin and coagulating milk were much alike in other characteristics.

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COLONY TYPES AND VARIETIES OF *PSEUDOMONAS AERUGINOSA*(Grown on nutrient agar, 3 days old. Magnification $\times 15$.)

FIG. 1. Green fluorescent colony of type A.

FIG. 2. Variety of type A green fluorescent colony demonstrating one or more depressed areas.

FIG. 3. Flat, spreading variety of type A green fluorescent colony. The entire colony is similar to the periphery of the type A colony.

FIG. 4. Type B colony responsible for the production of pyocyanin.

FIG. 5. A rough brown pigment-producing colony variety of type B.

FIG. 6. A white colony variety of type B.

FIGS. 7, 8, and 9. Type R colonies responsible for the production of the "Pyo" compounds.

Růžicka (1899) separated his cultures of the fluorescent organisms into two groups on the basis of their cultural, morphological, and physiological characteristics, one related to *Pseudomonas aeruginosa* and the other to *Pseudomonas fluorescens-liquefaciens*. Niederkorn (1900) studied 15 strains of the fluorescent microorganisms in an attempt to find better methods for differentiating the various forms. He concluded from these studies that there were only two constant forms, *P. aeruginosa* and *P. fluorescens-liquefaciens*, and that all of the others are varieties of these. Eisenberg (1914) also reported that the fluorescent microorganisms in the group are closely related. He found it difficult to separate them into species and varieties. Tanner (1918) reported that of a group of 100 strains of fluorescent organisms isolated from water, 4 produced endospores, but the rest constituted a homologous group, differences appearing only in regard to liquefaction of gelatin, formation of H_2S , and perhaps fermentation of glycerol.

Meador, Robinson, and Leonard (1925) concluded from their investigation that all typical strains of *P. aeruginosa* produce three water-soluble pigments: (1) fluorescent pigment, (2) pyocyanin, and (3) pyorubin. The pyocyanin and fluorescent pigments act as indicators, but pyorubin remains unaffected in color when treated with acid or alkali. These authors found that the majority of the fluorescent microorganisms which are derived from nature and which are culturally identical with *P. aeruginosa* will produce both pyocyanin and pyorubin on appropriate media and should therefore be classified as *P. aeruginosa*. These authors also reported that agglutination and absorption tests failed to reveal any differences among the various strains tested. Standiford (1937) found that he could not distinguish between *P. aeruginosa* and *P. fluorescens* culturally or serologically; thus he labeled all his strains *P. aeruginosa*. Brooks, Nain, and Rhodes (1925) examined a group of the fluorescent microorganisms associated with plant diseases by cultural, biochemical, and serological methods. By means of the cultural characteristics, the authors were able to arrange their bacteria into three groups: fluorescent, yellow, and white organisms. The biochemical reactions within the first two groups proved to be fairly constant, but preliminary serological examination of these strains revealed important groupings. Elrod and Braun (1942) observed that two cultures of *Phytomonas polycolor* were indistinguishable from certain strains of *P. aeruginosa* by biochemical and serological means. These authors, however, failed to confirm the view of Meador *et al.* that the *P. aeruginosa* group is serologically uniform. Reid and his associates (1942) studied 600 cultures, identified as *Pseudomonas*, and found in addition to serological differences monotrichic and lophotrichic types of flagellation, and differences in ability to grow at 37 C. These authors also described a mucoid phase.

Seleen and Stark (1943) found that the bacteria which produced a green fluorescent pigment were closely related. They divided 199 cultures of these microorganisms into 14 groups on the basis of their ability to grow at 5 C and 42 C, action on milk, liquefaction of gelatin, and reduction of nitrates. Subgroups were determined by the ability of the cultures to produce pyocyanin

and to utilize sucrose, acetic acid, lactic acid, or tartaric acid as the sole source of carbon. Tobie (1945) suggests that the genus *Pseudomonas* should comprise those rod-shaped microorganisms which produce water-soluble phenazine pigments.

ANTIBACTERIAL SUBSTANCES DERIVED FROM *PSEUDOMONAS AERUGINOSA*

As a result of the early and intensive studies of *P. aeruginosa*, it is not surprising to find that this microorganism was one of the first known to produce a substance antagonistic to other bacteria. Bouchard (1889) observed that the injection of small quantities of a culture of *P. aeruginosa* prevented the development of anthrax in rabbits that had been previously injected with a virulent strain of *Bacillus anthracis*. Woodhead and Wood (1889) found that sterilized cultures of *P. aeruginosa* had the same effect. Other investigators, Charrin and Guignard (1889), Emmerich and Low (1899), von Freudenreich (1888), and Blagovestchensky (1890), called attention to the fact that old cultures and culture filtrates of *P. aeruginosa* were bactericidal for many microorganisms.

The exact nature of this substance aroused a great deal of interest and controversy. Because of certain enzymatic activities of their product, Emmerich and Low named it "pyocyanase." Klimoff (1901) and Dietrich (1902), however, pointed out that the properties of this antibacterial substance were inconsistent with those of an enzyme. Later investigators, Raubitschek and Russ (1909), Ohkubo (1910), and Fukuhara (1911), reported that nearly all the bactericidal material could be extracted with lipid solvents. Thus, they attributed the antibacterial action to lipoids. Gundel and Wagner (1930), Hettche (1932, 1933, 1934), and Hettche and Vogel (1937) concluded that the antibacterial potency of the cellular extract was due to the fatty acid content.

In addition to pyocyanase, *P. aeruginosa* is known to produce another antibacterial substance. This is the blue-green, chloroform-soluble pigment, pyocyanin, the properties of which were investigated by Hettche (1932) and synthesized by Wrede and Strack (1924, 1928, 1929). From the chloroform extracts of *P. aeruginosa* cultures, Schoental (1941) isolated pyocyanin, alpha oxyphenazine, and a pale yellow oil, all possessing antibacterial properties.

Schoental's publication directed this laboratory's attention and interest to a study of the antibiotic materials occurring in cultures and filtrates of *P. aeruginosa*. The resources of the Departments of Bacteriology and Biochemistry were combined in order to investigate this problem in detail. A preliminary paper (Hays *et al.*, 1945) describes in detail the methods and materials used for production of the active materials. Crystalline, active substances have been isolated from crude alcoholic extracts of the cells of cultures five weeks old. These substances have been designated "Pyo" Ib, Ic, II, III, and IV in the chronological order of their isolation.

During the course of this investigation numerous bacteriological problems arose. Foremost among these was the desirability of obtaining a strain capable of producing a large and constant yield of the "Pyo" compounds. An effort was made, therefore, to determine the dissociative activities of *P. aeruginosa*.

Apparently little interest or attention has been directed toward the morphological appearance of the colonies of *P. aeruginosa*, and the few descriptions available are at variance one with another (Tanner, 1918; Ford, 1927; Kramer, 1935; Topley and Wilson, 1937; Park and Williams, 1939; Bergey *et al.*, 1939; Jordan and Burrows, 1942). However, Lartigau (1898), Hadley (1927), and Kramer (1935) called attention to the fact that *P. aeruginosa* is subject to variation under conditions of laboratory cultivation. Hadley observed that many cultures lose their ability to produce the chloroform-soluble, blue-green pigment. When such cultures are plated out, it was found that all of the colonies did not produce the blue-green color, but some merely possessed a yellow fluorescent pigment. Kramer further observed that the colony morphology of *P. aeruginosa* is also subject to variation. It appears fairly certain to us that this variation in pigment production alone accounts for much of the confusion encountered in the literature concerning the identification and classification of this microorganism. It is also possible that, with the change in pigment production, there may be associated changes in other characteristics as well. Therefore, a detailed examination of the various colony forms was undertaken in an attempt to determine the possible range of variability, if any, and also to catalogue those features which may characterize the appearance of the typical *P. aeruginosa* colony.

METHODS

Terminology. When the fluctuation in the generic and species terminology is considered, it seems rather futile to attempt to standardize the nomenclature of the dissociative subdivisions occurring in the species. The term "strain" is satisfactory only when referring to a stable daughter colony possessing characteristics different from that of the parent colony. The term "clone" is an excellent one when referring to the stable progeny of a single cell, and its use should not be abused. A review of the literature reveals a general overlapping in the meaning of all the popular terms, the choice of which is apparently left entirely to the investigator. Therefore, in this paper the term "type" will be used to refer to the cultures derived from selected colonies of the parent culture either by means of artificial selection or by means of dissociative activities. The term "variety" will be used in reference to any visible, external, dissociative tendencies occurring in the colony type. Thus we have genus, species, type, and variety.

Cultures employed. The *Pseudomonas aeruginosa* cultures were obtained directly from infected lesions whenever possible; thus, any changes or dissociations that developed could be accurately evaluated.

P-SLU, a stock strain maintained in our laboratory for the past several years, originally derived from a pathological lesion. A strong pyocyanin producer.

P-CC, isolated from a chronic bladder infection. A mediocre pyocyanin producer when first isolated, but this property was soon lost.

P-B, isolated from an infected burn. A strong pyocyanin former.

P-M, isolated from an abscessed tooth. Produces an abundance of pyocyanin.

P-U, *P-U1*, *P-U2*, *P-U3*, cultures received as *P. aeruginosa*, isolated from urine. These cultures produced an abundance of fluorescein but no pyocyanin.

P-S, isolated from a blood culture of a patient suffering from septicemia. A strong pyocyanin producer.

P-Ky, stock culture from the University of Kentucky. The culture produced no pigment.

P-KyA, stock culture from the University of Kentucky. A strong producer of pyocyanin.

P-9027, obtained originally from the A.T.C.C. This culture had recently been grown on lettuce leaves.

Throughout this investigation all colony studies were made on plain nutrient agar using the streak plate method. This method was found to be superior to the pour plates, as only surface colonies were desirable and it was more convenient for the handling of a large number of cultures. All incubations were at 37 C. Previous experience indicated that the plates should be incubated for 72 hours to ensure complete development of all colonies and to permit the appearance of any dissociative tendencies which might be present. Shorter periods of incubation may result in immature colonies and thus give rise to false interpretations; if incubated for longer periods, secondary or daughter colonies may develop. A small hand lens mounted on a ring stand was found to be very satisfactory for examining the colonies.

As culture *P-CC* was being employed routinely for the production of the "Pyo" compounds, this culture was used for most of the preliminary studies. When streak plates were made for a comparative study of the colonies, *P-CC* presented such a confusing variety that it appeared on first inspection to be contaminated. The colonies varied from blue-green in color to white, and from small, round, convex, opaque, to large, rough, spreading, flat, translucent forms. The ability of this culture to produce pyocyanin was slight, and this property was soon lost altogether.

With the aid of the lens a representative colony form of each major type present was selected, emulsified in nutrient broth, and restreaked on nutrient agar. These various colony types were followed through numerous generations in this manner until the respective types were 100 per cent reproducible or stable. This procedure was complicated by the fact that various colonies would lose their ability to produce pigment, not only pyocyanin, but fluorescent pigment as well. The latter may not be an actual loss in the true sense of the word, but rather it is possible, by colony selection, to separate from the fluorescent colony type a nonfluorescent variety that is identical in every other respect with the parent.

RESULTS

Colony types. As a result of these studies, it soon became apparent that the culture (*P-CC*) was composed of three basic colony types, which have been, for the lack of better terminology, called types A, B, and R.

Type A colonies appear as circular, smooth or undulate, convex; with trans-

lucent centers; effuse, flat, wavy, transparent periphery; and irregular, lobulated edges. These are the yellow fluorescent pigment producers and the predominant colony type of the culture.

Type B colonies are small, round, convex, translucent, and finely granular with entire edges. This colony type produces the blue-green pigment, pyocyanin. Frequently type B colonies are contained in and completely masked by type A colonies.

Type R colonies were somewhat variable; usually round or slightly irregular and curled, raised, or flat; umbilicate or umbonate; finely or coarsely granular; and at times almost filamentous. Edges might be entire, undulated, lobate, or auriculated. Although inconsistent in their morphological appearance, these variations were not sufficient to warrant new or different types.

Numerous combinations of these types may occur with corresponding colony configurations. It is also possible by appropriate means, such as animal passage, growing in high concentrations of glycerol, or repeated transfers in increasing concentration of homologous antiserum, to convert one colony type into another. Furthermore, each of the three basic colony types is subject to variation. Type A may be forced to throw off an A-depression variety, similar to the type A colony but exhibiting one or more depressed areas in the center. Type B may be forced to throw off a stable B-rough-brown variety, a coarsely granular, convex colony with irregular edges, and producing a diffusible, dark brown, chloroform-insoluble pigment.

The colony morphology of cultures *P-M*, *P-B*, and *P-S* is identical with that of *P-CC*. The urine cultures, *P-U*, *P-U1*, *P-U2*, and *P-U3* exhibited only type A colonies. *P-Ky* was a stable, nonpigmented variety of a type B colony, whereas *P-Ky1* was an unstable rough variety of type A. Culture *P-9027* was made up of stable type A colonies. Culture *P-SLU*, originally employed for the production of the "Pyo" compounds, but the use of which was discontinued when the crude extracts failed to produce the usual yield of active material, was made up of the typical type B colonies, and a large, smooth, spreading B variety. Types A and R were entirely lacking from this culture.

From these observations it seems plausible to assume that cultures derived from naturally occurring human infections are made up of more than one colony type. Whether these types were naturally occurring, or whether they originated from the dissociation of only one type, is debatable. The latter is probably more correct as shown by the fact that the colony types are subject to dissociation.

The derived varieties are reproducible from generation to generation by transferring carefully selected colonies, but they are by no means stable or permanent. The three basic colony types remain stable under ordinary conditions of laboratory cultivation, but dissociation occurs in old cultures. Occasionally permanent types or varieties are thrown off, i.e., a type B colony has been obtained that remains stable with 100 per cent of the colonies producing the blue-green pigment. Mouse passage, for which animal this particular type is highly fatal, resulted only in intensifying the pigment production.

Colonies exhibiting a "lytic" action similar to that described by Hadley (1924) were observed. This lytic action appeared only in the pyocyanin-producing B type colonies. With the aid of a straight needle, portions of these pitted areas were inoculated into young normal colonies and also onto fresh agar plates. This property was found to be nontransferable to the young colonies and reproducibility on fresh agar was extremely uncertain. It seems very unlikely that this action was due to a bacteriophage, although this possibility was by no means extensively investigated. It was also of interest to note that at various times numerous G type colonies would appear. These extremely small colonies could not be explained (crowding was not a factor) nor were they reproducible. These colonies are mentioned only to point out the extreme variations found.

When the colonies are transferred to nutrient broth, growth is more or less typical of the type. The B types are facultative anaerobes which grow uniformly throughout the medium with very little pellicle formation. When the tube is shaken the pellicle breaks up into very small fragments with little tendency to settle to the bottom. Type A, a strict aerobe, forms a heavy pellicle with only slight turbidity throughout the medium. When shaken the pellicle tends to break up into two or more clumps and to settle to the bottom. Type R, also a strict aerobe, grew only on the surface with the formation of a dense, almost mucoid, wrinkled pellicle. When shaken the pellicle forms a gummy, sticky mass which tends to adhere to the sides of the tube or flask just above the surface of the medium, but when shaken free will settle to the bottom. A new pellicle forms rapidly. Although type R produced a somewhat mucoidlike growth in broth and on agar, these organisms, including types A and B, did not produce capsules, nor did they give the slightest indication of encapsulation under any conditions.

Biochemical characteristics of the various colony types. The biochemical characteristics of these microorganisms are likewise extremely variable and few authors seem to agree on their fermentation patterns. The complexity of their extremely active endoenzyme and exoenzyme systems is such that classification of these organisms by this means alone is impossible. All varieties of *P. aeruginosa* studied will grow in a simple synthetic medium and will utilize glycerol or glucose as the sole source of carbon. In addition to a hemolytic enzyme, an active and independent proteolytic enzyme is produced; indole is not formed from tryptophane; and nitrates may or may not be reduced to nitrites. All types and varieties, with one exception, produced acid without gas from glucose and glycerol, whereas others produced acid without gas from glucose, glycerol, xylose, and galactose. One variety of culture *P-SLU* did not ferment any of the test substances.

An attempt was made to correlate fermentation pattern with colony types and varieties. The results recorded in table 1 indicate that while a slight correlation may be possible, it is by no means complete. It should be emphasized that these biochemical reactions are somewhat difficult to repeat. All of the culture characteristics listed in the table represent the average of a number of repeated tests employing young cultures. This lack of uniformity leaves one

TABLE 1
Biochemical reactions

CULTURE	VARIETY OR TYPE	GELATIN	LITMUS MILK	GLUCOSE	XYLOSE	GALACTOSE
<i>P-CC</i>	B	L 14 days	C 72 hr	+	+	+
	A	L 24 hr	D 48 hr	+	+	+
	R	L 24 hr	D 48 hr	+	+	+
	B	L 7 days	C 72 hr	+	—	—
	Brown	L 24 hr	D 72 hr	+	+	+
	A	L 24 hr	D 72 hr	+	+	+
	Dep.	L 24 hr	D 72 hr	+	+	+
<i>P-B</i>	B	L 14 days	D 72 hr	+	+	+
	A	L 24 hr	D 72 hr	+	+	+
	R	L 24 hr	D 48 hr	+	+	+
	B	L 14 days	D 7 days	+	—	—
<i>P-SLU</i>	Large	—	—	—	—	—
	B	L 14 days	D 14 days	—	—	—
	A	L 24 hr	D 48 hr	+	+	+
<i>P-S</i>	B	L 72 hr	D 96 hr	+	+	+
	R	L 24 hr	D 48 hr	+	+	+
	A	L 24 hr	D 96 hr	+	—	—
	B	L 24 hr	D 72 hr	+	+	+
<i>P-M</i>	A	L 24 hr	D 48 hr	+	+	+
	R	L 24 hr	D 48 hr	+	+	+
	A	L 48 hr	D 72 hr	+	—	—
	B	L 24 hr	D 72 hr	+	+	+
<i>P-9027</i>	A	L 48 hr	D 72 hr	+	—	—

L = Liquefaction; C = Curd; D = Digestion; + = Acid production; — = No reaction.

in doubt as to a means of identifying this microorganism. It is, therefore, convenient and certainly not subject to very severe criticism to consider those bacteria which are gram-negative, nonsporeforming, possessing 1 to 8 polar

flagella, actively proteolytic and hemolytic, producing a blue-green or fluorescent pigment (whether or not chloroform-soluble), and producing acid without gas from glucose, as belonging to the species *Pseudomonas aeruginosa*.

Antigenic pattern. Meader, Robinson, and Leonard (1925) reported variations in the agglutinin content of different antisera, but absorption with any of their cultures resulted in complete reduction of both homologous and heterologous reaction. They concluded, therefore, that the group is serologically uniform. Aoki (1926) concluded from his experiments that the microorganisms are antigenically dissimilar. Harris (1940), Naghski (1941), and Reid *et al.* (1942) reported "M" and "S" phases of the organism. The "M" or encapsulated phase was naturally occurring, but the "S" phase was induced by cultivation in homologous immune sera. Their agglutination and cross-agglutination tests indicated that there was a close antigenic relationship of the organisms in the Dawson "M" phase, but that old stock cultures were not so antigenically similar to freshly isolated cultures. The microorganisms in the "S" phase indicated a homologous serological relationship. Harris reported three distinct serological types in the Dawson "M" phase. Elrod and Braun (1942) and Munoz, Scherago, and Weaver (1945) found the group to be serologically heterologous.

In this investigation, antisera were prepared against each of the basic colony types and varieties of culture *P-CC*, and of cultures *P-SLU*, *P-Ky*, *P-U*, and *P-9027*. Both formalin- and alcohol-treated cells were prepared. Formalin is known to preserve and even increase to some extent the agglutinability of motile bacteria, and alcohol destroys the agglutinability of the H antigen; but the O antigen retains its antigenic properties. The antisera were prepared by suspending the growth of 18-hour agar cultures in formalinized saline and injecting rabbits every other day by the intravenous route with increasing dosages of the cell suspension. The amount of antigen per injection was found to be of secondary importance, but it was desirable to obtain the maximum antibody production in the minimum length of time to prevent broadening of the antigenic pattern. Dosages starting with 0.25 ml and doubling with each subsequent injection until 2 ml were reached were found to give satisfactory titers as shown by trial bleedings from the ear artery. Following this series of injections, living organisms were given in the same manner with a resulting slight increase in titer. Subcutaneous injections of the antigen are satisfactory, but the time factor is increased and the antibody titers are, on the whole, slightly lower.

Because of the variability of these colony types, it was desirable to prepare sufficient quantities of the antigens to last throughout the investigation. Agglutination, cross-agglutination, and agglutinin-absorption tests were then run in an attempt to correlate the various colony types. All H agglutinations were incubated in the water bath at 55 C for 2 hours, although agglutination was usually complete in less than 30 minutes. The O agglutinations were incubated at 55 C for 8 hours and overnight at icebox temperature. The method of adding the cell suspension to the decreasing concentrations of antisera was entirely arbitrary. The results obtained by adding one drop of a heavy cell suspension to each tube were identical with those observed when one ml of a thin cell sus-

TABLE 2
"O" type agglutination

AGGLUTINATING CELLS	ANTISERA					
	A-P-CC	B-P-CC	R-P-CC	A-P-U	A-P-9027	P-SLU
A-P-CC	1,280*	320	1,280	1,280	640	640
B-P-CC	1,280	320	1,280	640	1,280	1,280
R-P-CC	1,280	320	1,280	1,280	1,280	640
A-P-U	1,280	160	1,280	1,280	640	640
A-P-9027	640	320	640	320	1,280	320
P-SLU	320	320	320	320	160	1,280
A-P-U1	1,280	160	1,280	1,280	320	640
A-P-U2	1,280	160	1,280	1,280	320	640
A-P-U3	1,280	160	1,280	1,280	320	640
A-P-B	1,280	320	1,280	1,280	640	640
B-P-B	640	320	640	1,280	640	1,280
R-P-B	1,280	320	1,280	1,280	1,280	640
A-P-M	1,280	160	640	640	1,280	640
B-P-M	1,280	160	640	1,280	640	1,280
A-P-S	1,280	160	640	640	1,280	640
B-P-S	640	160	640	640	640	1,280

* Figures represent the highest dilution showing 4 plus agglutination.

TABLE 3
"H" agglutination

FORMALIN- TREATED CELLS	ANTISERA								
	A-P-CC	B-P-CC	R-P-CC	A-P-9027	P-Ky	P-SLU	A-Dep. P-CC	B-Br. P-CC	A-P-U
A-P-CC	>10,240	1,280	>10,240	2,560	320	320	>10,240	5,120	>10,240
B-P-CC	5,120	5,120	>10,240	320	>10,240	>10,240	>10,240	>10,240	1,280
R-P-CC	>10,240	1,280	>10,240	640	>10,240	2,560	>10,240	>10,240	2,560
A-P-9027	2,560	80	320	>10,240	40	1,280	80	80	640
P-Ky	20	40	<20	<10	>10,240	<20	<20	<20	<20
P-SLU	640	1,280	640	320	160	>10,240	160	320	320
A-Dep. P-CC	>10,240	1,280	>10,240	640	>10,240	160	>10,240	5,120	>10,240
B-Br. P-CC	160	5,120	2,560	320	>10,240	320	>10,240	>10,240	320
A-P-M	>10,240	640	5,120	640	>10,240	320	>10,240	2,560	>10,240
B-P-M	2,560	5,120	5,120	320	>10,240	>10,240	5,120	>10,240	1,280
R-P-M	>10,240	1,280	>10,240	640	>10,240	1,280	5,120	5,120	1,280
A-P-B	>10,240	640	5,120	2,560	>10,240	160	>10,240	1,280	2,560
B-P-B	2,560	5,120	5,120	320	>10,240	>10,240	5,120	5,120	1,280
R-P-B	>10,240	5,120	>10,240	2,560	>10,240	160	>10,240	1,280	5,120
A-P-U	>10,240	640	5,120	1,280	>10,240	320	>10,240	1,280	>10,240
A-P-S	>10,240	640	5,120	1,280	>10,240	320	>10,420	2,560	>10,240
B-P-S	2,560	5,120	2,560	160	>10,240	1,280	5,120	>10,240	1,280

pension was added. It is to be observed in tables 2 and 3 that specific O and H agglutinins were demonstrable in the blood serum of rabbits inoculated

with formalinized cell suspensions. The flagellar agglutinins may be absorbed out, leaving the somatic agglutinins unaffected (table 4). It is to be further observed that reciprocal agglutinin absorption showed that the O types are serologically identical regardless of the colony types. If one should consider only the somatic agglutinogens and their respective agglutinins, the results would indicate that the *P. aeruginosa* group is serologically homologous. Differences are apparent only when testing for the flagellar agglutinogens. Even then, as is evident in table 3, the group appears more or less homologous. Culture *P-Ky* was entirely out of line with the other types, there being no correlation whatsoever. However, it would seem from the agglutinin content of its anti-serum that the flagella or cell contains complete agglutinogens but that these were present in such small quantities that they were not demonstrable under the conditions of the test. Neither could culture *P-Ky1* be correlated with the other colony types. This culture is not included in the tables.

TABLE 4
"O" agglutination

ALCOHOL-TREATED CELLS	B-P-CC ANTISERUM ABSORBED WITH "H" CELLS OF				A-P-U ANTISERUM ABSORBED WITH "H" CELLS OF			
	A-P-CC	B-P-CC	R-P-CC	A-P-U	A-P-CC	B-P-CC	R-P-CC	A-P-U
A-P-CC	320	320	320	320	1,280	1,280	1,280	1,280
B-P-CC	320	320	320	320	640	640	640	640
R-P-CC	320	320	320	320	1,280	1,280	1,280	1,280
A-P-U	160	160	160	160	1,280	1,280	1,280	1,280

The absorption of A-P-CC and R-P-CC antisera with the "H" cells had no effect on the agglutinability of the alcohol-treated cells. Both antisera agglutinated to titer, i.e., 1,280.

A summary of the results of the flagellar agglutinin-absorption tests is presented in table 5. It is evident that the three colony types, A, B, and R, are serologically heterologous when tested in this manner. The majority of these types are not pure, that is, they contain some agglutinogens of heterologous types. This is particularly true when the colony types have been derived from the same culture. However, it is possible that pure types or varieties could be developed by continuous colony selection and transfer, or by single cell isolation.

"Pyo" production. As the original purpose of this investigation was partly to obtain a culture of *P. aeruginosa* capable of producing greater and more constant yields of the "Pyo" compounds, each experimentally developed colony type was investigated for its ability to produce these antibiotic materials. As P-CC was known to be a satisfactory producer of "Pyc," each of the colony types from this culture was inoculated into routine culture carboys. The types used were known to remain stable for at least 2 weeks when grown in a liquid medium in the test tube. The growth of each type was carefully followed by streaking samples on nutrient agar at weekly intervals. It was always desirable to know the type of organism contained in the pellicle as well as the type growing

throughout the medium. Type B remained stable during the 5-week period. Because it was a facultative anaerobe, growth was abundant throughout the

TABLE 5
Agglutinin-absorption reaction

AGGLUTINATING CELLS	A-P-CC ANTISERUM ABSORBED WITH "H" CELLS OF					
	A-P-CC	A-P-U	A-P-B	B-P-CC	B-P-B	R-P-CC
A-P-CC	<40	<40	<40	2,560	10,240	320
A-PU	<40	<40	<40	10,240	10,240	1,280
A-PB	<40	<40	<40	5,120	1,280	1,280
A-P-M	<40	<40	<40	10,240	10,240	2,560
A-P-S	<40	<40	<40	5,120	10,240	2,560
A-P-9027	<40	<40	<40	1,280	1,280	1,280
B-PCC	<40	80	<40	<40	<40	640
B-P-B	<40	40	<40	<40	<40	640
B-P-S	<40	80	<40	<40	<40	1,280
R-P-CC	<40	160	80	640	5,120	<40
B-P-CC ANTISERUM ABSORBED WITH "H" CELLS OF						
	B-P-CC	B-P-M	B-P-B	A-P-CC	A-P-U	R-P-CC
B-P-CC	<40	<40	<40	640	2,560	320
B-P-M	<40	<40	<40	1,280	1,280	1,280
B-P-B	<40	<40	<40	2,560	1,280	640
B-PS	<40	<40	<40	2,560	1,280	1,280
B-Brown						
P-CC	<40	<40	<40	640	1,280	320
A-P-CC	<40	<40	<40	<40	<40	640
A-P-U	<40	<40	<40	<40	<40	1,280
A-P-B	<40	<40	<40	<40	<40	1,280
R-P-CC	<40	<40	<40	320	1,280	<40
R-P-CC ANTISERUM ABSORBED WITH "H" CELLS OF						
	R-P-CC	R-P-M	R-P-S	B-P-CC	A-P-CC	A-P-M
R-P-CC	<40	<40	<40	1,280	640	1,280
R-P-M	<40	<40	<40	5,120	1,280	1,280
R-P-S	<40	<40	<40	5,120	1,280	2,560
R-Spreading						
P-CC	<40	<40	<40	1,280	320	1,280
B-P-CC	<40	<40	<40	<40	2,560	5,120
B-P-M	<40	<40	<40	<40	5,120	1,280
A-P-CC	<40	<40	<40	640	<40	<40
A-P-M	<40	<40	<40	1,280	<40	<40
A-Dep-						
P-CC	<40	<40	<40	640	160	320

medium with only scant pellicle formation. When the carboy was shaken, the pellicle broke up into numerous pieces and had little tendency to settle to the bottom. Extraction of these 5-week-old cells yielded approximately one-fourth

of a satisfactory antibacterial titer. Type A did not remain stable during the 5-week period; both B and R types were thrown off. The antibacterial titer was found to be directly related to the number of R type organisms present in the pellicle. The carboy inoculated with type R produced a very satisfactory yield of "Pyo." This fact was confirmed by inoculating routine batches of carboys (24 carboys per batch). The growth was typical of the R type with a heavy, wrinkled, slimy pellicle and with scant growth throughout the medium. Similar experiments employing types from other cultures conclusively proved that type R was responsible for the production of "Pyo."

DISCUSSION OF THE RESULTS

Cultures of *P. aeruginosa* isolated from infections in man have been shown to vary considerably in the morphological appearance of their colonies. By the transfer of selected colonies it has been possible to demonstrate that these numerous variants are derived from at least three basic colony types. For a lack of better terminology these types have been designated A, B, and R. By a variety of means it has also been possible to demonstrate that any one of these basic types may be transformed into another type. There is also sufficient evidence to support the view that the dissociation of these organisms may occur *in vivo* as well as under various environmental conditions *in vitro*. It is of interest to note that the colonies of type A described above are indistinguishable from those of *P. fluorescens* as described elsewhere, and it is quite possible that they are identical. This same theory might well account for many of the recently described species occurring in the genus *Phylomonas* that are serologically indistinguishable from *P. aeruginosa*. In view of the experimental evidence presented it seems reasonable to assume that a great deal of the present confusion encountered in the literature concerning this species can be explained adequately by the dissociative behavior of the various cultures studied.

The morphological appearance of the typical *P. aeruginosa* colony is subject to considerable discussion. If the ability of a culture to produce pyocyanin continues to be an essential criterion for the identification of *P. aeruginosa*, then we must consider the type B colony described above as typical of the species. However, the fluorescent colonies of type A were in general more numerous, and it is the opinion of this investigator that the experimental evidence points to this colony type as being typical of the species. On the other hand, the typical colony morphology may be of only minor importance when one considers the dissociative ability of the species and the tendency to lose its ability to produce the blue-green pigment, pyocyanin. A comparative examination of stock cultures that have been carried for a number of years in the laboratory will further prove the dissociative behavior of *P. aeruginosa*, not only in the morphological appearance of the colonies but also in their biochemical characteristics as well. The biochemical characteristics of these microorganisms are entirely unreliable and, with the exception of the reactions in gelatin, litmus milk, and glucose, are apparently of little importance. The organisms of types A and R were, on the whole, more versatile than those of type B.

With regard to the serological pattern of *P. aeruginosa* it is evident that this species is serologically heterologous if we consider that the culture contains a variety of colony types. However, similar colony types obtained from various cultures proved to be serologically identical. Therefore, it is reasonable to assume that the various conflicting results which have been reported are due to the heterologous colony types employed in the various studies. One also finds that the majority of the authors are not specific in stating whether the O or H type of agglutination was observed; thus the type can only be assumed from the agglutinating titers which they have recorded. If this be true, then some of the investigators were no doubt studying the somatic antigens and others were investigating the flagellar antigens. Naturally, the two sets of results appear contradictory, but, in view of the evidence presented above, both may actually be correct. The somatic antigens are serologically homologous, but agglutinin-absorption tests show a definite heterologous relationship existing between the flagellar antigens of the three basic colony types. The flagellar agglutinogens of identical colony types, however, are serologically homologous.

The "M" phase of *P. aeruginosa*, described by Reid and his associates, was not observed in this investigation. Colonies of the R type may be described as somewhat mucoidlike, but the organisms were entirely devoid of capsules.

SUMMARY

The results of a study of the dissociation of *Pseudomonas aeruginosa* have been reported. By appropriate means it was possible to demonstrate that colonies derived from cultures of *Pseudomonas aeruginosa* isolated from human infections vary considerably in their morphological appearance. These colony variations were numerous and were shown to be derived from at least three basic colony types designated herein as types A, B, and R.

The patterns of fermentative and proteolytic activities of these colony types were extremely variable and apparently would be of little value as a means of classification.

Agglutination reactions of the various cultures of *P. aeruginosa* indicated that the somatic antigens are homologous, but agglutinin-absorption tests show a definite heterologous relationship existing between the flagellar antigens of the three basic colony types.

The types of *P. aeruginosa* responsible for the production of the "Pyo" compounds and pyocyanin have been identified and described.

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OBSERVATIONS ON STRAINS OF A MONOPHASIC SALMONELLA VARIANT¹

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The first known isolation of the *Salmonella* type having the antigenic formula IV, V, XII: e, h- was made in 1941 by Dr. D. W. Bruner from the intestine of a hog affected with enteritis; in the list of Standard Strains of *Salmonella* (Edwards and Bruner, 1942) this monophasic e, h variant is assigned no. 150. On the basis of the slow fermentation of rhamnose and an unconfirmed report that cultures containing O-factor V could be recovered from the original *S. reading* strain (no. 19), the monophasic organism was considered to be a variant of *S. reading*. This designation may have been an error in nomenclature since subsequent developments suggest that the variant may have been derived from one of several, perhaps commoner, *Salmonella* types.

During 1942 and 1943 six additional representatives of this variant were isolated in California and submitted to the National Salmonella Center at Lexington for verification; more recently, three more strains were received from California. In April, 1944, the organism was recognized in a routine stool specimen taken at the Naval Medical School, and from January through May, 1945, twenty-nine cultures were isolated in various Naval establishments and subsequently submitted to the Enteric Pathogen Laboratory for identification; three of the latter were duplications so that only twenty-six individual strains are represented.

This recent accumulation of a rather remarkable number of members of the monophasic variant group led to a restudy of the epidemiologic, biochemic, and serologic characteristics of the cultures in the hope that some clue to the ancestry of the type might be found. For the purpose of completeness the available data on the 36 strains are listed below.

150 (Hog 24682)—isolated in 1941 by Bruner from intestine of hog affected with enteritis.

CDAI 157—isolated in May, 1942; CDAI 222 and CDAI 223—isolated in June, 1942; CDAI 363 and CDAI 368—isolated in May, 1943; from poults on a California turkey ranch.

¹ The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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Cal. 605—Isolated in March, 1943, by Los Angeles County General Hospital Laboratory from the feces of a seven-month-old baby suffering with acute gastroenteritis.

CDAI 989—isolated in June, 1945, from a turkey in Lancaster, California.

CDAI 998 and CDAI 999—Isolated in June, 1945, from turkeys in Hemet, California.

100-73—Isolated in April, 1944, by NAMRU no. 2 from a stool specimen submitted to the Naval Medical School; no further information available.

106-10—Isolated in January, 1945, from the feces of a patient on the USS Orion at Pearl Harbor during an outbreak of gastroenteritis; turkey was incriminated as the vehicle of dissemination.

106-24—Isolated in March, 1945, from the skin of a frozen turkey in a lot that was transferred in November, 1944, from the supply ship USS Mizar to the USS Orion at Woendi.

106-44—Isolated in March, 1945, from a man on the USS Orion at Pearl Harbor during continued investigation of the January outbreak; clinical and epidemiological data not available.

106-70—Isolated in March, 1945, from a mild case of enteritis in man during the continued investigation of the January outbreak on the USS Orion.

106-126, 106-128, 106-129, 106-130, 106-131, 106-132, 106-133, 106-134, 106-135, 106-136, and 106-137—All isolated in April, 1945, from the feces of patients during an outbreak of gastroenteritis on the USS Courtland at Pearl Harbor; all had histories negative for previous diarrheal disease.

106-141 and 106-142—Isolated from the feces of two food handlers during the April outbreak on the USS Courtland; men were asymptomatic at the time but had typical symptoms in March, 1945, during a small epidemic aboard the ship.

106-145—Isolated from the feces of a food handler during the April, 1945, outbreak on the USS Courtland; was asymptomatic at the time and denied any history of previous diarrheal disease.

106-236—Isolated in March, 1945, from the feces of a food handler at Bachelor Officers' Quarters in Mokalapa, T. H.; no further information available.

10-273—Isolated in January, 1945, in the laboratory of the U. S. Naval Hospital at Pearl Harbor from the feces of a rheumatic fever patient who had fever and diarrhea.

10-274—Isolated in January, 1945, in the laboratory of the U. S. Naval Hospital at Pearl Harbor from the feces of a gastroenteritis patient stationed on the USS Baham; an outbreak of enteritis involving at least 21 men occurred on this ship in October, 1944.

10-275—Isolated in January, 1945, in the laboratory of the U. S. Naval Hospital at Pearl Harbor from the feces of a gastroenteritis patient; no further information available.

10-277—Isolated in January, 1945, in the laboratory of the U. S. Naval

Hospital at Pearl Harbor from the feces of a case diagnosed as duodenal ulcer, but with no symptoms of diarrheal disease.

82-111—Isolated in May, 1945, at a California Naval Station from the feces of a neuro-psychiatric patient who gave a history of mild diarrhea in August, 1941, while in Kamuela, T. H.

103-5—Isolated in March, 1945, from the feces of a Marine patient on Iwo Jima whose staging area was in Hawaii; case diagnosed as intestinal hemorrhage and had other symptoms of diarrheal disease; no information available on previous history.

It is unfortunate that scant information is available concerning the circumstances surrounding the original sources of several of these cultures. The first isolation (no. 150) was from a hog affected with enteritis, but it is not known whether other animals in the same herd were sick. Eight of the strains were isolated from poult and turkeys in California, but no data are available as to whether the birds were ill. Attempts have been made to determine whether the turkey from which culture 106-24 was isolated may have been procured in California, but so far the information has not been obtained.

In man, these e,h variants are capable of causing symptoms of gastroenteritis comparable with those incited by other *Salmonella* types. Some strains were from allegedly asymptomatic subjects with no history of diarrheal disease; others were found in mild cases; and still others were from individuals who exhibited moderately severe symptoms. At present, no known cases of enteric fever have occurred. In general, the symptoms described are fever of about 103° F, diarrhea with 10 stools per day, nausea, vomiting, and abdominal cramps; the duration of symptoms is about 2 days and the average stay on the sick list is approximately 5 days. A few cases may be ill enough to require sedation and intravenous fluids.

Although one culture (Cal. 605) was isolated from an apparent sporadic case and others may have been single cases, the available evidence also shows that under certain somewhat obscure conditions epidemics may be initiated. In one outbreak aboard a ship in the Pacific, 19 cultures were isolated from cases with acute symptoms; 4 cultures were recovered from individuals asymptomatic at the time. Three of these 4 individuals were food handlers, and 2 of them were ill with typical symptoms during an acute outbreak aboard the same ship a month previously. It seems possible that these two food handlers may have remained carriers following the first outbreak and could have been responsible for initiating the second epidemic. The organism was involved in an outbreak of gastroenteritis on another ship in the Pacific, but 3 other *Salmonella* strains, including a newly described type, *S. orion* (Barnes *et al.*, 1945), plus a concurrent shigellosis, complicated the picture.

The monophasic e,h strains appear to be endemic in California and the Pearl Harbor area of Hawaii, but further information is necessary before the geographic area from which the variants originated can be determined. At the time culture 106-24 was isolated, several other frozen turkeys from the Naval Supply Depot,

Fremantel, West Australia, were cultured but no strains of *Salmonella* were found. The original source of the Fremantel turkeys is not available.

BIOCHEMICAL CHARACTERISTICS

All cultures studied exhibited the morphological and tinctorial attributes of the genus *Salmonella*. In view of the delayed fermentation of rhamnose observed in studying the original culture (no. 150) of these e,h variants (Edwards and Bruner, 1942), an extensive re-examination of the biochemical characteristics of the 36 strains was undertaken in the hope of detecting some significant disparity that might suggest possible ancestry. The results are summarized in table 1.

Remarkably consistent behavior is apparent from the biochemic study of these cultures; the only variations noted were in rhamnose, melibiose, and cellobiose. No culture was observed to ferment rhamnose in less than 2 days, but all cultures produced acid by the eighth day; 14 of the 36 strains produced a small volume of gas from rhamnose, but the remainder were grossly anaerogenic. Two cultures (CDAI 157 and 150) that initially required 6 and 7 days, respectively, to produce acid detectable in 1 per cent rhamnose broth containing Andrade's indicator were serially transferred in this medium, and following the first or second subculture both strains produced both acid and gas within 24 hours. This fact suggests that the delayed and incomplete fermentation of rhamnose first observed may be due to a predominance in the original culture of organisms possessing sluggish enzymes and that selection or adaptation of cells with more active rhamnose-splitting enzymes may result in more prompt and complete fermentation.

With the exception of 3 strains all cultures produced acid and gas in melibiose within 24 hours. Acid and gas production by CDAI 368 was observed first on the seventh day and by 10-273 and 106-24 on the third day. After 2 serial transfers in 1 per cent melibiose CDAI 368 produced acid and gas within 24 hours.

In cellobiose all cultures exhibited delayed activity; the time required for visible evidence of fermentation varied from 3 to 6 days. A single strain (106-10) remained anaerogenic during the period of observation, but in several instances gas production was not evident for 1 or 2 days after acidity was noticeable.

The increased rate of fermentation of a given carbohydrate subsequent to serial transfer of *Salmonella* strains in the medium is well known. Although no definite evidence is at hand of an alteration in antigenic composition correlated with a change in enzymatic activity of these organisms, the possibility of the two variations occurring coincidentally should not be overlooked.

If it is assumed that the monophasic e,h strains under discussion were derived from some established *Salmonella* type as a result of a "loss variation" in antigenic composition, the most likely ancestors would appear to be in the group composed of *S. reading* (19), *S. chester* (17), *S. san-diego* (110, 111), *S. saint-paul* (108), *S. zagreb* (132), *S. kaapstad* (145), *S. kaposvar* (133), and *S. salinatis* (148). In order to test this hypothesis from the biochemic standpoint, 22 strains of *S. saint-paul* were inoculated into rhamnose broth. Ten of the cultures were isolated from 6 patients during an epidemic on board a ship in the Pacific, but the

TABLE 1

Biochemical characteristics of 36 strains of a monophasic Salmonella variant

	MANNITOL, GLUCOSE, XYLOSE, MALTOSE, DULCITOL, ARABINOSE, TREHALOSE, SORBITOL	RHAM- NOSE	CELLO- BIOSE	MELI- BIOSE	LACTOSE, SUCROSE, INOSITOL SALICIN ADONITOL, MELEZITOSE	UREA, GELATIN, V. PROS- KAUER, INDOLE, i-TAR- TRATE	STERN, H ₂ S, MUCATE, CITRATE, d-TAR- TRATE, MOTILITY, METHYL RED	PURPLE MILK	TRIMETHYLAMINE- OXIDE	i-TARTRATE
10-273	AG ₁	A ₅	A ₄ G ₆	AG ₃	—	—	+	N	+	—
10-274	AG ₁	A ₃	AG ₄	AG ₁	—	—	+			
10-275	AG ₁	AG ₅	AG ₄	AG ₁	—	—	+			
10-277	AG ₁	AG ₃	A ₄ G ₆	AG ₁	—	—	+			
82-111	AG ₁	AG ₅	A ₄ G ₆	AG ₁	—	—	+	N		
100-73	AG ₁	A ₂	AG ₄	AG ₁	—	—	+	N		+
103-5	AG ₁	AG ₅	A ₄ G ₆	AG ₁	—	—	+	N		
106-10	AG ₁	A ₃	A ₃	AG ₁	—	—	+	N	+	+
106-24	AG ₁	A ₄	AG ₄	AG ₃	—	—	+	N	+	+
106-44	AG ₁	A ₅	AG ₅	AG ₁	—	—	+	N	+	+
106-70	AG ₁	A ₄	AG ₄	AG ₁	—	—	+	N	+	+
106-126	AG ₁	A ₅	A ₆ G ₇	AG ₁	—	—	+			
106-128	AG ₁	AG ₆	AG ₄	AG ₁	—	—	+			
106-129	AG ₁	A ₆	A ₆ G ₇	AG ₁	—	—	+			
106-130	AG ₁	A ₃	A ₄ G ₆	AG ₁	—	—	+			
106-131	AG ₁	A ₆	AG ₄	AG ₁	—	—	+			
106-132	AG ₁	AG ₅	A ₄ G ₆	AG ₁	—	—	+			
106-133	AG ₁	A ₅	A ₄ G ₆	AG ₁	—	—	+			
106-134	AG ₁	A ₅	A ₄ G ₆	AG ₁	—	—	+			
106-135	AG ₁	A ₄	A ₄ G ₆	AG ₁	—	—	+			
106-136	AG ₁	A ₄	A ₄ G ₆	AG ₁	—	—	+			
106-137	AG ₁	A ₅	AG ₄	AG ₁	—	—	+			
106-141	AG ₁	A ₆	AG ₄	AG ₁	—	—	+			
106-142	AG ₁	A ₃	AG ₄	AG ₁	—	—	+			
106-145	AG ₁	A ₆	A ₄ G ₆	AG ₁	—	—	+			
106-236	AG ₁	A ₅	AG ₄	AG ₁	—	—	+			
24682	AG ₁	A ₇	AG ₄	AG ₁	—	—	+	N	+	+
CDAI 157	AG ₁	A ₆	AG ₆	AG ₁	—	—	+			+
CDAI 222	AG ₁	A ₄ G ₇	A ₄ G ₆	AG ₁	—	—	+	N		+
CDAI 223	AG ₁	A ₄ G ₇	A ₄ G ₆	AG ₁	—	—	+			+
CDAI 363	AG ₁	AG ₅	AG ₄	AG ₁	—	—	+			+
CDAI 368	AG ₁	AG ₄	A ₄ G ₆	AG ₇	—	—	+			+
CDAI 989	AG ₁	AG ₅	A ₂ G ₄	AG ₁	—	—	+			+
CDAI 998	AG ₁	A ₄ G ₆	AG ₄	AG ₁	—	—	+			+
CDAI 999	AG ₁	A ₄ G ₆	A ₄ G ₆	AG ₁	—	—	+			+
CAL. 605	AG ₁	A ₄ G ₆	A ₄ G ₆	AG ₁	—	—	+	N		—

Subscripts indicate number of days of incubation before reaction was apparent. Where no gas production is indicated, it was not apparent after 21-30 days' incubation.

Negative in carbohydrates = no acid or gas after 21-30 days at 37 C.

Urea negative = no ammonia production after 1-2 days at 37 C.

Gelatin negative = no liquefaction after 29-37 days at 37 C.

V-P negative = no acetylmethylcarbinol production after 24-28 hours at 37 C.

Indole negative = no production after 3 days at 37 C.

i-Tartrate negative = no utilization after 14 days at 37 C.

TABLE 1—*Continued*

Sterns positive = pale or deep violet during first 3 days of incubation.

H₂S positive = strong hydrogen sulfide production during first 24 hours at 37 C.

Mucate positive = Complete utilization during incubation at 37 C for 14 days.

Citrate positive = same as mucate.

d-Tartrate positive = same as mucate.

Motility positive = active motility.

Methyl red positive = pronounced acid reaction in presence of methyl red indicator following incubation at 37 C for 4 days.

N = characteristic *Salmonella* reaction of initial acidity followed by return to neutrality and subsequent increasing alkalinity. 11 cultures tested.

Trimethylamine oxide positive = marked production of trimethylamine within 24 hours. 6 cultures tested.

l-Tartrate + = complete utilization during incubation at 37 C for 14 days. — not utilized during incubation at 37 C for 14 days. 15 cultures tested.

balance were from widely separated geographic areas inside and outside the United States. In addition, 10 strains of *S. san-diego* from well-distributed sources were cultured in the same carbohydrate medium. All cultures fermented rhamnose overnight with the production of acid and gas.

Of interest are some observations concerning the fermentation of inositol. Nine strains of *S. saint-paul*, 2 strains of *S. chester*, and 1 of *S. kaapstad* all produced acid and gas promptly from this carbohydrate, but all 36 strains of the e,h variant, 5 strains of *S. san-diego*, and 1 strain each of *S. zagreb* and *S. kaposvar* failed to show any activity in the medium during the period of observation. It was noted, however, that, whereas the diphasic strain of *S. reading* (19) produced acid and gas in inositol, the monophasic e,h strain (150) produced no visible change. Such results appear to be consistent with a hypothesis that the ancestor of the e,h variants may be one of the 8 types mentioned. If this were the case, the focus of attention might be returned to *S. reading* on the basis of a correlated biochemic and serologic loss variation. This is not intended to infer, however, that *S. san-diego* or *S. saint-paul*, for example, should be excluded from consideration.

The utilization of certain organic acids was partially investigated. One strain of the e,h variant (Cal. 605) utilized *d*-tartrate, but not *l*-tartrate to a significant degree; this was an unexpected result, but observations with other cultures and the hypothetical precursors in *d*-, *l*-, and *i*-tartrate failed to reveal anything else noteworthy.

In general the biochemic studies failed to produce much encouragement in the search for an ancestor of the monophasic e,h variants; the observations do not, however, lead to an exclusion of any of the 8 diphasic types mentioned as possibilities.

SEROLOGICAL CHARACTERISTICS

The most direct method for designating the parent type of the e,h variants would be to isolate and identify a second flagellar phase from at least one of the monophasic strains. Another procedure entails the actual observation of the

ancestor in the process of loss variation; this would obviously depend to a large extent upon chance.

A considerable amount of work has been done in both laboratories in attempts to demonstrate serologic relationship. All of the monophasic variants possess the somatic antigens IV, V, XII and the flagellar phase 1 complex e,h. Isolation of a second flagellar phase was attempted soon after receipt of the cultures. The method consisted in cultivating the organisms in semisolid medium (Edwards and Bruner, 1942) containing an amount of sterile, single-factor h serum adequate to suppress motility in phase 1 (e,h) but of insufficient concentration to immobilize a second phase if present (Gard, 1937). This procedure was later repeated with 29 of the 36 strains using larger inoculums; incubation at 37 C extended for 10 to 15 days. Under these conditions no evidence was obtained of the existence of a second phase. The same 29 cultures were cultivated in petri plates of the same medium containing an appropriate amount of h serum in order to determine if the increased surface would favor the separation of a second phase; these cultures also remained monophasic.

Seven of the cultures (150, CDAI 157, CDAI 222, CDAI 223, CDAI 363, CDAI 368, and Cal. 605) have been studied intensively in the Lexington laboratory as a part of a series of investigations on monophasic variants in the genus *Salmonella*. These strains have been cultivated in semisolid medium containing h antiserum for 1 year with biweekly transfers; also, all cultures were kept on agar slants for 1 year with monthly transfers. Cultures 150, CDAI 157, CDAI 222, and CDAI 223 were grown in broth containing 1:1,000 concentration of pure h serum and e,n. . . vaccine, and also in broth containing h serum and 1,2,3. . . vaccine. The vaccines were heavy suspensions from agar slants devitalized by heating at 58 C for 30 minutes; they flocculated well in homologous serum and were added in 0.1-ml amounts to tubes containing 2.0 ml of broth. The serum-vaccine-broth mixtures were inoculated with 1 loopful of a 1:100,000-dilution of a broth culture of the organism being tested; they were transferred at 5-day intervals and tested at each transfer for phase variation. Under none of these circumstances did a second phase appear.

Formalinized, motile broth cultures of all the monophasic variants flocculated well in a 1:1,000-dilution of e,h serum; when tested in suitable dilutions of single-factor serums a strong reaction occurred with h serum but not with n serum. Two of the strains from man (106-130 and Cal. 605) and one from turkey (CDAI 989) were used to test the extent of adsorption of agglutinins from the strong e,h serum prepared from the original monophasic hog culture 24682 (150). The results showed that all 3 cultures completely exhausted e,h agglutinins from the serum, thus indicating that the e,h phases of these 4 variants are very closely related if not identical. The same 3 cultures were employed in adsorbing the somatic antibodies IV, V, XII from *S. typhi-murium* (9) serum that initially agglutinated culture 106-130 to a dilution of 1:1,000. After adsorption by any one of the 3 strains (106-130, Cal. 605, CDAI 989) the *S. typhi-murium* serum failed to react in a 1:50-dilution with the usual alcohol-heat-treated antigens of any one of the 3 adsorbing strains or the homologous *S. typhi-murium*. The

O antigens of these monophasic strains are, therefore, very closely related to if not identical with those of *S. typhi-murium*.

The serologic studies here reported have, therefore, failed to shed any light on the derivation of the monophasic e,h variants.

DISCUSSION

The first known strain of the monophasic e,h *Salmonella* was isolated from a hog with enteritis in 1941. Subsequently the organism was recovered from turkeys on California ranches and on board a Naval ship in the vicinity of Pearl Harbor, T. H. It has also been isolated from the feces of an acute case of enteritis in man in California, and, more recently, a remarkably increased number of strains were isolated from Naval personnel in the Central Pacific. Some of the latter were asymptomatic at the time cultured (at least one of these denied any history of diarrheal disease); others were apparently sporadic cases of gastroenteritis; and still others were cases representing epidemics. Of further epidemiologic importance is the fact that food handlers were represented in the two categories, asymptomatic carriers and acute cases. Under these circumstances organisms of this type cease to be simple biologic curiosities and assume major public health significance as incitants of salmonellosis. The symptomatology of the infections is similar to the syndrome observed in other acute salmonelloses. Of the infections in man so far reported an appreciable number appear to have had their immediate origin in a lot of turkeys obtained from a Naval supply ship in the Pacific. At present, information as to the original source of these turkeys is not at hand. Evidence indicates, however, that this *Salmonella* type has been endemic on certain turkey ranches in California. Recently obtained data suggest also that the infection has been endemic in man in the Hawaiian area and at times becomes epidemic. An important epidemiologic principle will be demonstrated if subsequent information shows that the organisms were introduced into the Central Pacific by means of contaminated turkeys from California. Nothing is known regarding the source of the strain isolated at Bethesda, but it is entirely possible that the subject had duty either in California or Hawaii or both.

The abrupt increase in the number of typed strains of this organism and evidence of its widened dissemination revived interest in attempts to designate a diphasic ancestor of this monophasic variant. It is believed that the type developed as a result of "loss variation" in some other *Salmonella*, either known or as yet undescribed, and that the organism has become stabilized in monophasic form. Observations herein reported were concerned with the biochemic and serologic characteristics of 36 strains of the type. From the serologic standpoint, 8 established *Salmonella* types were considered as possible precursors of the variant because of their possession of the e,h complex in flagellar phase 1 and somatic antigens IV, XII or IV, V, XII. It is biologically possible, however, that the e,h factors were acquired by some other type through a process somewhat analogous to that described by Griffith (1928). This approach should be in-

vestigated further. The serologic observations described failed to shed any light on the question.

During a comprehensive restudy of the biochemic characteristics of the 36 e,h variants in comparison with those of some of the possible ancestors, particular attention was paid to the fermentation of rhamnose and inositol. The activity in rhamnose of the various organisms studied did not yield any new evidence, but an observation in inositol deserves comment. It was noted that in inositol the diphasic strain of *S. reading* (19) produced acid and gas, but the monophasic strain (150) was apparently inert. This finding is not inconsistent with a working hypothesis that the monophasic e,h type may have been derived from one of the diphasic organisms through a process of correlated variation (Topley and Wilson, 1938). The answer to this question will, however, have to await the discovery of more direct evidence, which may, perhaps, be obtained by chance observations.

SUMMARY

Attention is called to a recently observed increase in the prevalence and distribution of a monophasic *Salmonella* type with the antigenic formula IV, V, XII: e,h-. The organisms have been recovered from swine, fowls, and in man from asymptomatic carriers, sporadic cases, and epidemics of gastroenteritis. The strains appear to be endemic in California and Hawaii. It is suggested that the designation *S. reading* for this organism should be subjected to further scrutiny until more direct evidence of its probable diphasic ancestry is acquired. Certain of the clinical and epidemiological characteristics of infections due to this group of organisms were described. Extensive biochemic and serologic experiments cited were designed to detect, if possible, some clue as to the derivation of the monophasic variants. Serologic results were not fruitful, but the suggestion is made that the strains may have developed as a result of a biochemic variation correlated with an antigenic alteration. Some possible further lines of approach are mentioned.

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A METHOD FOR DIFFERENTIATING CANDIDA ALBICANS IN TISSUE

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During a study of the distribution and staining properties of *Candida albicans* (Robin) Berkhout, 1923, in tissues of inoculated animals, we realized that fat staining might be tried. This had been done with blastospores from culture media but not from organs. As a result of our study, we found that fat staining of tissue blastospores not only gave information about certain aspects of *C. albicans* metabolism in the parasitic state, but helped in its identification in tissues by giving better structural details.

As alcoholic Sudan is employed in current histological techniques, we tried it for staining the fat of blastospores, but satisfactory results were not obtained. Then we successfully tested the same stain dissolved in lactic acid, which has been used for fat staining in blastospores from culture media. After use of Sudan III, we found that Sudan IV (scarlet red) gave even more beautiful pictures.

Five minutes were found the optimal time for staining with lactic acid Sudan, though a shorter time gave acceptable results. For counterstaining the tissue harboring *C. albicans*, toluidine blue gave better differentiation than other stains. As a control, we employed culture cells treated by the same method.

TECHNIQUE

The thinnest possible frozen sections are stained 5 minutes in 0.1 per cent scarlet red in pure lactic acid. After washing in distilled water, sections are placed for 2 minutes in 1 per cent aqueous toluidine blue, then washed carefully and mounted in Apathy's fluid or in a saturated glucose solution. Examination is preferably made with the oil immersion objective. The solution of scarlet red in lactic acid must be fresh and filtered before use, as it tends to form precipitates. Old solutions give rise to artifacts very similar to fat droplets.

Rabbit kidneys and rat prostates obtained from animals inoculated with a strain of *C. albicans* of known pathogenicity were used. Some of the specimens had been in 10 per cent formaldehyde for more than one year; others had been fixed for several hours. No difference was found between old and fresh specimens.

RESULTS

Fat was readily visible in any *C. albicans* cells having fat. Fatty degeneration of tissue was also much better demonstrated with lactic acid Sudan than with alcoholic Sudan.

The shape of the blastospores was the same as that of those seen in cultures, though no budding was observed and the size was generally smaller. The protoplasm was colorless or, in a few, slightly stained blue. The fat stained orange in the characteristic shape and distribution. The technique gave beautiful pictures of unstained blastospores with orange fat inclusions contrasting sharply with the blue tissue background. The frozen tissue sections were not altered by our technique. A few hyphae developing from blastospores were seen with stained fat inclusions.

As Gram's method is regularly employed for staining visceral fungi, we made an actual comparison of it with our technique. Gram-stained blastospores appeared solidly colored, some adopting bizarre shapes, a few with clear spaces in the protoplasm, others broken, and several unstained. Possibly, the unstained forms correspond to those seen by us in vaginal smears treated by the same method. In these preparations, we have seen gram-negative young blastospores and, in the same filament, gram-negative and gram-positive segments alternating. In addition, in gram-stained tissues, the histological technique alters the blastospores and mycelia, which show a shape somewhat different from that of culture cells observed in fresh preparations. On the other hand, the Gram technique stains blastospores in a somewhat uniform way, whereas with the present method, the double enveloping membrane, the protoplasm, and its vacuoles and inclusions are sharply defined. In a word, our technique gives pictures of *C. albicans* in tissues which are practically identical with those of cultures.

SUMMARY

A simple and rapid technique is described for demonstrating *Candida albicans* (Robin) Berkhout, 1923, in animal tissues by staining the parasite's intracellular fat with Sudan IV dissolved in lactic acid, and counterstaining the tissue with toluidine blue. This method does not alter the shape nor the structure of blastospores and hyphae.

A METHOD FOR THE PREPARATION OF COMPLEMENT-FIXING ANTIGENS IN A STUDY OF EXPERIMENTAL TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS)

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Other investigators (Bengtson, 1944, 1945; Topping and Shepard, 1945) have recently reported the successful preparation of complement-fixing antigens of *Rickettsia orientalis*, grown in the yolk sac of fertile hens' eggs, that were suitable for use in the diagnosis of tsutsugamushi disease (scrub typhus fever). These findings have been confirmed by other workers as well as by ourselves (Cox *et al.*, 1945). However, the methods of preparation described in these reports are not too readily adaptable to large-scale production. Moreover, those authors have stated that their preparations contained only specially selected yolk sacs that showed large numbers of rickettsiae by microscopic examination (Topping, 1945).

In view of the results hitherto obtained it seemed advisable to attempt to develop a method that would be applicable to fairly large-scale production and that would utilize the yolk sacs from all living eggs harvested daily for the preparation of antigens of at least a moderately potent titer. In other words, yolk sacs showing only moderate or even poor rickettsial growths would be utilized, and the processing procedure should be of such a nature as to conserve and concentrate all antigenic substances present.

This paper describes a method of preparing complement-fixing antigens that seems to meet the foregoing requirements.

MATERIALS AND METHODS

Rickettsial strain. The Karp strain of *Rickettsia orientalis*, obtained through the courtesy of Dr. Norman H. Topping of the National Institute of Health and with the permission of the United States of America Typhus Commission, was used throughout the experiments.

Source of infectious material. With a few exceptions, all antigens were prepared from infected yolk sac membranes of fertile hens' eggs inoculated as described by Cox (1938), and were processed from daily harvests containing from 300 to 900 eggs a harvest. A few antigens were prepared from other portions of the fertile eggs, such as the yolk fluid and the chorio-allantoic membrane, but, as will be shown later, such preparations were not nearly so active as those prepared from yolk sac membranes. Twelve to thirty-six yolk sacs were examined microscopically from each lot of eggs on the eighth, ninth, tenth, or eleventh day after inoculation, and the results of these

samplings were considered as representative of the whole lot and determined the time of harvesting of all remaining, living eggs.

The majority of the antigen preparations were prepared from yolk sacs that showed an average of at least "one plus" rickettsial growth. Such a numerical evaluation was determined in the following manner: Smears showing rickettsiae varying in numbers from 1 up to 6 or 8 per oil immersion field were termed "positive"; smears showing from 6 to 8 up to a countable number of rickettsiae per field were termed "one plus"; and smears showing uncountable numbers of rickettsiae in nearly every field were termed "two plus." It must be emphasized that in no instance could the *average count* of the yolk sac harvests concerned in this report be considered as "two plus" growth.

Preparation of antigens. A general outline of the method used for preparing the antigens is as follows:

(1) Infected yolk sacs (either freshly harvested or those kept frozen *in toto* in the CO₂ icebox at -70°C and later thawed in a water bath at about 37°C) were weighed and homogenized in a Waring blender to a 50 per cent tissue suspension with the addition of an equal volume of distilled water containing phenol to 0.8 per cent concentration.

(2) The resulting 50 per cent tissue suspension was held for at least 48 hours in the cold room at 4°C.

(3) The inactivated suspension was dispensed into freezing bottles of the blood plasma type, shell-frozen in a dry ice alcohol mixture, and completely dried by evacuation from the frozen state.

(4) The shell of dried tissue was well broken up in the bottle by means of a spatula and the material transferred to an Erlenmeyer flask.

(5) One volume of diethyl ether (based on the original amount of 50 per cent tissue suspension employed) was added to the flask, and the contents were well mixed. The flask was tightly stoppered and allowed to stand at 4°C for 3 to 4 hours with occasional shaking.

(6) The ether was separated from the tissue mass by means of a Buchner funnel fitted with two layers of coarse (crepe) filter paper. Small particles of tissue adherent to the walls of the extraction flask were collected by rinsing the flask with small portions of fresh ether and adding the washings to the mass in the funnel. After being freed of all excess ether by suction, the tissue was carefully scraped from the funnel in small thin flakes and replaced in the extraction flask.

(7) Step no. 5 was repeated except that 1½ to 2 volumes of fresh ether were used. The extraction with ether was again carried out for 3 to 4 hours or even overnight at 4°C with occasional shaking.

(8) Step no. 6 was repeated, and the dry, pulverized material was then transferred to thick-walled, pyrex glass bottles suitable for the subsequent removal of the remaining ether by high vacuum. A simple apparatus consisting of a cenco-megavac pump and an ether trap immersed in a dry ice alcohol bath served to remove traces of residual ether by allowing the material to remain under high vacuum for 6 to 18 hours at room temperature.

(9) The dried powder was weighed and placed in a bottle containing a few sterile glass beads. A quantity of saline (containing phenol to 0.3 per cent con-

centration), sufficient to resuspend the material to a volume equal to that of the original 50 per cent tissue suspension, was added. The suspension was stored at 4 C for 3 to 5 days with occasional shaking each day.

(10) The suspension was centrifuged at 3,000 rpm for 30 minutes at room temperature in an International, size I centrifuge equipped with an angle head rotor. The sediment was discarded. The resulting supernatant fluid constituted the antigen.

Various modifications were made in this procedure in an attempt to secure antigens of higher complement-fixing titers, and these will be noted later in describing each antigenic preparation.

Each preparation was evaluated by the results obtained in complement-fixing tests in which the antigens were titrated in the presence of a known scrub-typhus-immune guinea pig serum.

Complement-fixation test. For the complement-fixation tests a modified Kolmer and Boerner (1941) technique was employed using 2 units of amboceptor, 2 exact units of complement, and a 2 per cent suspension of washed sheep cells.

In the titration of the antigen, 0.25 ml of diluted, inactivated serum from guinea pigs recovered from tsutsugamushi disease were added to 0.25-ml amounts of antigen that had been serially diluted 2-fold. Two exact units of complement in a volume of 0.5 ml were then added to each tube and the tubes held in the cold room at 4 C overnight. The following morning the hemolytic system (consisting of 2 units of hemolysin in a volume of 0.25 ml plus 0.25 ml of a 2 per cent suspension of washed sheep cells) was added, and the tubes were kept at 37 C until the control tubes cleared. Readings were then made immediately.

In the determination of the titer of tsutsugamushi disease antiserum, 0.25 ml of antigen containing approximately 3 antigenic units were added to 0.25-ml amounts of inactivated serum that had been serially diluted 2-fold. Two exact units of complement (determined in the presence of antigen) in a volume of 0.5 ml were then added to each tube and the tubes held in the cold room at 4 C overnight. The method of adding the hemolytic system and of incubating the tubes at 37 C before the reading was the same as that used in determining the antigenic titer.

The pool of tsutsugamushi-disease-immune serum, against which all the antigen preparations were tested, was obtained from a group of convalescent guinea pigs that had been inoculated with infected mouse liver suspensions of the Karp strain. This serum gave a complement-fixing titer of "4 plus" in a dilution of 1:128 and in all antigenic titration tests was used in a dilution of 1:20.

EXPERIMENTAL

The effect of prolonged contact with phenol upon the crude tissue suspension. Living yolk sacs were harvested from over 900 eggs on the tenth day after inoculation and stored frozen *in toto* at -70 C in a CO₂ box for 3 to 6 weeks. The yolk sacs were then processed as shown above except that step no. 2¹ was modified as follows: Phenol (0.4 per cent concentration) was allowed to act on

¹ These numbers refer to the steps involved in the processing procedure outlined under the section *Preparation of antigens*.

one portion of the crude tissue suspension for 2 days, on a second for 12 days, and on a third for 33 days before continuing with the refining process.

The complement-fixation results obtained with these antigens (table 1) indicate that 0.4 per cent phenol, in contact with the crude tissue suspension for as long as 33 days, exerts no appreciable deleterious effect upon the final product.

Comparative antigenicity of various portions of the infected fertile egg. The yolk sacs, chorio-allantoic membranes, and yolk fluid were harvested separately from 8 eggs that were still living on the tenth day after inoculation.² The various

TABLE 1

Effect of prolonged contact with phenol upon crude tissue suspension

ANTIGEN SERIES	PROCEDURE NOTES	YOLK SAC CONCENTRATION OF FINAL PRODUCT	ANTIGEN DILUTION TESTED						ANTIGEN CONTROLS		
			2	4	8	16	32	64	2	4	8
K-11-Aa*.	2 days in phenol	25 per cent	4	4	4	1					
K-11-B .	12 days in phenol	50 per cent	4	4	4	4	2				
K-11-C ..	33 days in phenol	50 per cent	4	4	4	4	2				

* This antigen was resuspended to a volume equivalent to a 25 per cent yolk sac suspension for other purposes of study. From data presented later (series K-16, table 6) it is reasonable to assume that if the concentration of this antigen had been 50 per cent instead of 25 per cent the complement fixation results would have been identical to those of antigens K-11-B and K-11-C.

TABLE 2

Comparative antigenicity of various portions of the infected fertile egg

ANTIGEN	EGG MATERIAL	ANTIGEN DILUTIONS TESTED						ANTIGEN CONTROLS		
		2	4	8	16	32	64	2	4	8
K - 12 - A	Yolk sac membrane	4	4	4	4	4	1	4	1	
K - 12 - B ..		4	4	1				4		
K - 12 - C ..	Yolk fluid	4	1							

portions were then processed as outlined above except for steps no. 1 and no. 2. No. 1: The membranes and fluid were prepared as 10 per cent suspensions by weight in 0.4 per cent phenolized saline. No. 2: The suspensions were all stored at 4 C for 40 days before processing. After being processed each preparation was resuspended to a volume calculated to be equivalent to that of an original 50 per cent tissue suspension.

The data in table 2 show that when these various preparations were titrated

² The embryos were likewise harvested separately but an accident prevented completion of the antigen. This experiment is being repeated.

as antigens the yolk sac proved to be at least 8 times more active than the chorio-allantoic membrane or yolk fluid.

The effect of inactivating the rickettsiae with ether or phenol and preserving the antigens with formalin or phenol. Yolk sac membranes were harvested from 310 living eggs on the tenth day after inoculation and were held frozen *in toto* at -70°C for 2 to 3 days. The yolk sac suspensions were then processed as outlined above except for steps no. 1 and no. 10. No. 1: One half of the 50 per cent tissue suspension was inactivated by the addition of 0.4 per cent phenol, and the other half was inactivated by the addition of 10 per cent ether by volume. No. 10: Both preparations, after ether extraction, were resuspended in saline containing 5 per cent ether. To one half of each preparation phenol was added to 0.1 per cent concentration, but to the other half of each preparation was added 0.1 per cent formalin.

TABLE 3

The effect of inactivating the rickettsiae with ether or phenol and preserving the antigens with formalin or phenol

ANTIGEN	PROCEDURE NOTES	RESULTS SOON AFTER PREPARATION						RESULTS AFTER 6 WEEKS' STORAGE AT 4°C					
		2	4	8	16	32	64	2	4	8	16	32	64
K-16-B	Ether-killed, formalin-preserved	4	4	4	4	1		4	4	1			
K-16-C	Ether-killed, phenol-preserved	4	4	4	4	1		4	4	4	3		
K-16-J	Phenol-killed, phenol-preserved	4	4	4	4	2		4	4	4	3-4		
K-16-K	Phenol-killed, formalin-preserved	4	4	4	4	3		4	4	2			
K-11-C	(Used as a control)	4	4	4	4	2		4	4	4	3-4		

Table 3 shows the results obtained when the various preparations were titrated as antigens soon after their preparation and again after 6 weeks' storage at 4°C .

The data show that no appreciable differences could be discerned in the antigens when they were tested soon after their preparation. When tested after 6 weeks' storage, however, all showed a decrease in titer. The preparations preserved with phenol showed the least drop in titer—approximately 2-fold—but those preserved with formalin showed the greatest loss in activity—approximately 4-fold.

The preparations inactivated with phenol gave slightly higher titers than those inactivated with ether, but the differences were not great enough to be considered significant.

The antigenicity of yolk sac membranes harvested from dead embryos. Yolk sac membranes were harvested from embryos that had died at various intervals after inoculation. The yolk sac suspensions were processed as outlined except for steps no. 1 and no. 2. No 1: The yolk sac membranes were homogenized to a 50

per cent suspension in 0.85 per cent salt solution containing 0.25 per cent phenol. No. 2: The suspensions were held at 4 C for 10 to 15 days before processing.

The data in table 4 indicate that yolk sacs harvested from embryos dead on the eighth to tenth day after inoculation demonstrate some activity when tested as antigens. However, their activity was so poor when compared with that of

TABLE 4
The antigenicity of yolk sac membranes harvested from dead embryos

ANTIGEN	PROCEDURE NOTES	ANTIGEN DILUTIONS TESTED				ANTIGEN CONTROLS		
		2	4	8	16	2	4	8
DD-4...	Dead 4 days after inoculation							
DD-5	Dead 5 days after inoculation							
DD-6	Dead 6 days after inoculation							
DD-7...	Dead 7 days after inoculation							
DD-8	Dead 8 days after inoculation	3						
DD-9	Dead 9 days after inoculation	4	1					
DD-10.	Dead 10 days after inoculation	4	3					

TABLE 5
The effect of various lipid solvents upon antigenicity

ANTIGEN	SOLVENT	EXTRACTION TEMPERATURE	ANTIGEN DILUTIONS TESTED						ANTIGEN CONTROLS		
			2	4	8	16	32	64	2	4	8
K-15-A	Ether	4 C	4	4	4	4	4				
K-15-B	Alcohol	4 C	4	4	4						
K-15-C...	Acetone	4 C	4	4	4	4	4	1	1		
K-15-D...	Toluene	26 C	4	4	4	4	4				
K-15-E	Chloroform	26 C	4	4	4	4	3				
K-15-F...	Benzene	26 C	4	4	4	4	3				
K-15-G.	Ethyl acetate	26 C	4	4	4	4	3				

TABLE 6
The quantitative nature of the method for preparation of antigens

ANTIGEN	PER CENT OF SUSPENSION IN TERMS OF ORIGINAL TISSUE	ANTIGEN DILUTIONS TESTED						ANTIGEN CONTROLS		
		2	4	8	16	32	64	2	4	8
K-16-E.....	10	4	4	1						
K-16-F....	25	4	4	4	1					
K-16-G	50	4	4	4	4	1				

the antigens prepared from living eggs that it seemed advisable to discard all dead embryos.

The effect of various lipid solvents upon antigenicity. Yolk sac membranes were harvested from 300 living embryos on the tenth day after inoculation and held frozen *in toto* at - 70 C for 10 to 30 days. The frozen and dried material was divided into 7 equal portions and these were processed as outlined except for

steps no. 5 and no. 7 wherein various other fat solvents were employed in the same volume as ether.

The data in table 5 show that, with the single exception of alcohol, all the fat solvents gave antigen preparations as potent as that obtained by using ether.

TABLE 7

Specificity tests of antigen K-11-C

Antigen titration in the presence of various antirickettsial immune serums

SERUM NO.	CONVALESCENT GUINEA PIG SERUM	HOMO- LOGOUS TITER OF IMMUNE SERUM	IMMUNE SERUM DILUTION	ANTIGEN DILUTIONS TESTED					
				2	4	8	16	32	64
K-3.....	Tsutsugamushi disease (Karp)	1:256	1:20	4	4	4	4	1	
Ep. typhus no. 21 ...	Epidemic typhus (Breinl)	1:128	1:10	2	1				
End. typhus no. 2-10 ..	Endemic typhus (Wilmington)	1:512	1:10	2	1				
RMSF no. 3.	Rocky Mt. spotted fever (McCullough)	1:512	1:10	1					
Q9M no. 1.	American Q fever (Nine Mile)	1:128	1:10						

TABLE 8

Specificity tests of antigen K-11-C

Immune serum titrations in the presence of antigen K-11-C diluted 1:6 (3 antigenic units)

SERUM NO.	CONVALESCENT GUINEA PIG SERUM	SERUM DILUTIONS TESTED							
		4	8	16	32	64	128	256	512
K-3	Tsutsugamushi disease (Karp)	4	4	4	4	4	4	3	1
Ep. typhus no. 21	Epidemic typhus (Breinl)								
End. typhus no. 2-10	Endemic typhus (Wilmington)	1							
RMSF no. 3.	Rocky Mt. spotted fever (McCullough)								
Q9M no. 1.....	American Q fever (Nine Mile)								

Quantitative nature of the method for preparation of antigens. Yolk sac membranes were harvested from 310 living embryos on the tenth day after inoculation and held frozen *in toto* at -70°C for 2 to 3 days. The yolk sac suspension was processed as outlined except for step no. 10. No. 10: The dried and extracted material was divided into 3 portions and these were resuspended to volumes equivalent to 10, 25, and 50 per cent tissue suspensions.

The results in table 6 show that these antigens gave complement-fixing titers quantitatively directly proportional to the concentration of tissue used in their preparation.

Specificity of antigen. Approximately 1,200 ml of antigen K-11-C were prepared by the outline described above and this preparation was set aside as a reserve stock antigen. Specificity tests of this preparation were carried out against immune serums obtained from guinea pigs convalescent from epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever, American Q fever, and tsutsugamushi disease (scrub typhus), Karp strain.

Table 7 demonstrates the specificity of this antigen when it was titrated in the presence of various antirickettsial immune serums, and table 8 shows the results obtained when various antirickettsial immune serums were titrated in the presence of tsutsugamushi disease antigen K-11-C (Karp).

Comment

Various preparations similar to those noted in this report are being tested as vaccines to determine their immunizing capacities in experimental animals and man.

In studies to be reported later it has been determined that this method is readily applicable to the preparation of other rickettsial diagnostic antigens such as epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever, and American Q (Nine Mile) fever.

SUMMARY AND CONCLUSIONS

(1) A method is described for the preparation of complement-fixing antigens in a study of experimental tsutsugamushi disease (scrub typhus).

(2) The freezing and drying procedure employed in this method seems to possess several advantages over other methods used in the preparation of rickettsial complement-fixing antigens processed entirely in the fluid state:

(a) It is not necessary to use specially selected yolk sacs (based on microscopic examination) to prepare an antigen of practical value.

(b) Various lipid solvents, either miscible or immiscible with water, may be used partially or completely to extract the lipids from the yolk sac tissues without showing any appreciable adverse effect on the antigenic preparation.

(c) It is possible to work with yolk sac suspensions equivalent to 50 per cent tissue content and thus conserve and concentrate the antigenic components without resorting to further physical or chemical procedures.

(3) Complement-fixing antigens of *Rickettsia orientalis* prepared by this method do not show cross fixation with immune serums of epidemic (louse-borne) typhus, endemic (murine) typhus, Rocky Mountain spotted fever or American Q (Nine Mile) fever.

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THE COMPARATIVE SENSITIVITY OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS NEUTRALIZATION TESTS IN CHICK EMBRYOS AND IN MICE¹

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While neutralization tests in embryonated eggs have been described by a number of authors, the methods on the whole have been based on the quantitative ability of an immune serum to suppress the pocklike lesions produced by the free virus, and in only a few instances (Burnet and Galloway, 1934; Keogh, cited in Burnet, 1936; Higbie and Howitt, 1935) has death or survival of the embryos been used as a criterion of neutralizing capacity.

Since the Venezuelan equine encephalomyelitis virus is highly and uniformly lethal for chick embryos (Koprowski and Lennette, 1944), the possibility was considered that a neutralization test in this species, based on alterations of the lethal end point of the virus by a serum, might perhaps prove to be more sensitive than the usual intracerebral test in mice. Serum-virus mixtures, accordingly, were tested by parallel inoculation into eggs and mice, and a comparison of the results is presented here.

MATERIAL AND METHODS

An egg passage strain (Koprowski and Lennette, 1944) of virus was used. The test sera came from individuals recovered from laboratory infections with the Venezuelan equine encephalomyelitis virus (Lennette and Koprowski, 1943) and from rabbits immunized by repeated injections of formol-inactivated virus (Lennette and Koprowski, 1944) followed by living virus.

The neutralization tests were conducted as follows: Six infected chick embryos were ground in a previously chilled blender to a 20 per cent suspension in 10 per cent sheep serum broth and centrifuged at 3,500 rpm for 20 minutes in an electric centrifuge equipped with an angle head. The supernatant fluid was drawn off and used to prepare a series of decimal dilutions in serum broth.

The undiluted sera were distributed into tubes, and an equal volume of virus dilution was added. The tubes were shaken, and the mixtures were inoculated at once into eggs and into mice. Eggs were inoculated onto the chorio-allantoic membrane by the technique of Burnet and Faris (1942), Scotch tape being used in place of cover slips; for comparison, 28-day-old mice were inoculated intracerebrally. For control purposes and on the chance that the sensitivity of the egg test might approach that of the extraneural test in mice (Lennette and

¹ The work on which these observations are based was conducted with the support and under the auspices of the Serviço de Estudos e Pesquisas Sobre a Febre Amarela, which is maintained jointly by the Ministry of Education and Health of Brazil and the International Health Division of The Rockefeller Foundation.

Rabbit (no. 1)	None	Chick embryo	Normal Immune				5/6	2/6		5/6	4/6	1/6	0/6	10 ^{-9.25} 10 ^{-5.85}	3.40	3.20	6.55
		28-day-old mice i.c.	Normal Immune	6/6	5/6	5/6	6/6	4/6	6/6	6/6	3/6	0/6		10 ^{-9.00} 10 ^{-5.80}			
		21-day-old mice i.p.	Normal Immune	6/6	2/6	3/6	1/6	0/6	6/6	6/6	3/6	1/6	1/6	10 ^{-8.30} 10 ^{-1.75}			
Rabbit (no. 2)	1 hour at 37 C	Chick embryo	Normal Immune		6/6	6/6	6/6	6/6	1/6	6/6	1/6	1/6	0/5	10 ^{-8.70} 10 ^{-4.70}	3.00	4.00	
		28-day-old mice i.c.	Normal Immune	6/6	6/6	4/6	5/6	3/6	2/6	6/6	5/6	3/6	0/6	10 ^{-8.35} 10 ^{-4.35}			
Rabbit (no. 2)	None	Chick embryo	Normal Immune			6/6	5/6	4/6	6/6	1/6	1/6	3/6	1/5	10 ^{-9.15} 10 ^{-8.35}	2.80	3.40	5.50
		28-day-old mice i.c.	Normal Immune	6/6	6/6	6/6	3/6	1/6	6/6	6/6	1/6	1/6	1/6	10 ^{-8.70} 10 ^{-5.30}			
		21-day-old mice i.p.	Normal Immune	6/6	2/6	4/6	2/6	1/6	0/6	6/6	5/6	2/6	0/6	10 ^{-8.65} 10 ^{-3.15}			
Rabbit (no. 2)	1 hour at 37 C	Chick embryo	Normal Immune		6/6	6/6	6/6	6/6	5/6	2/6	0/6	1/6	1/6	10 ^{-8.80} 10 ^{-6.65}	2.15	3.80	
		28-day-old mice i.c.	Normal Immune	6/6	6/6	6/6	6/6	4/6	1/6	6/6	6/6	4/6	0/6	10 ^{-9.25} 10 ^{-5.45}			

Koprowski, 1944; Olitsky and Harford, 1938), the mixtures were also inoculated intraperitoneally into 21-day-old mice. Embryos and mice were used in groups of six for each serum-virus mixture, and the inoculum was held constant at 0.03 ml.

On completion of the inoculations, the mixtures were incubated in a water bath for 1 hour at 37 C in order to take advantage of any enhancement of neutralization which might occur during the lengthened contact of virus with serum, and then inoculated into a second series of eggs, and into mice intracerebrally. The incubated mixtures were not tested intraperitoneally, since the degree of neutralization demonstrable in the extraneural test is maximal and unaffected by incubation (Olitsky and Harford, 1938; Koprowski and Lennette, unpublished experiments; Morgan, 1945).

The inoculated eggs and the mice were examined daily for 10 days, and the total number of embryos and of mice dying within this period was used to compute the LD₅₀ titer (Reed and Muench, 1938) of the virus in the presence of immune and of normal serum. These titers are shown in table 1 under the heading "Effective virus titer"; the difference between the two titers is given in the last three columns of the table, according to the test species and route of inoculation employed, and represents the logarithm of the number of LD₅₀ of virus neutralized by an immune serum.

RESULTS

The results of the several neutralization tests are presented in table 1. It will be observed that the amount of virus neutralized by human immune serum no. 1 in eggs and by the cerebral route in mice was small, that it was approximately equal in both instances, the difference being less than twofold, and that incubation did not increase the neutralizing capacity.

Human immune serum no. 2 failed to give any significant neutralization in eggs, even after incubation of the serum-virus mixture. By the intracerebral test in mice, however, a definite neutralizing action was evident, and it was of about the same order with or without incubation of the mixture.

Both rabbit sera when tested without incubation of the serum-virus mixtures showed practically the same neutralizing capacity in eggs and by the cerebral route in mice. Following incubation, the neutralizing capacity of the sera was less in the egg test and greater in the mouse test, although the differences from the preincubation neutralizing capacity were small and within the range of experimental error. The cumulative effect of the decrease in the egg test and the increase in the corresponding mouse test, however, was such that the neutralizing capacity of serum no. 1 apparently was tenfold greater, and of serum no. 2 about fortyfold greater, in the mice than in the eggs; whether these differences, under the circumstances, can be regarded as significant is uncertain.

From the results as a whole, however, it appears that the sensitivity of these two tests is about the same, and hence the use of embryonated eggs offers no advantage over the use of mice. Neither test, as is shown in table 1, is as sensitive as the extraneural test in mice.

SUMMARY

The neutralizing capacities of Venezuelan equine encephalomyelitis immune sera were determined by inoculation of the same serum-virus mixtures onto the chorio-allantoic membrane of fertile eggs, and intracerebrally or intraperitoneally into mice. The results indicate that the sensitivity of the egg test is essentially similar to that of the intracerebral mouse test and much less than that of the extraneural mouse test.

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NOTE

A NOTE ON pH TOLERANCE OF AEROBACTER AEROGENES AND AEROBACILLUS MACERANS AS RELATED TO NATURAL ECOLOGY AND DECOMPOSITION OF ACID FOOD PRODUCTS

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Recently, two instances of spoilage of high-acid food products by bacteria generally considered as low-acid-tolerant types have been observed. In the first case, strains of *Aerobacter aerogenes* were found to decompose potassium bitartrate (cream of tartar) in liquid media having pH values of 3.9 to 4.2. In the other instance strains of *Aerobacillus macerans* were found to cause spoilage of canned fruits having original pH values of 3.8 to 4.0. Evidence for the unusual tolerance of both *Aerobacter aerogenes* and *Aerobacillus macerans* was obtained by repeated growth of the purified cultures in media of high acidity. All pH values were determined with the glass electrode.

The cultures of both *Aerobacter aerogenes* and *Aerobacillus macerans* were typical of the species as described in the literature. The strains of *Aerobacter aerogenes* were obtained from spoiled calcium tartrate and soil. The cultures of *Aerobacillus macerans* were obtained from water, canned peaches, and diced, mixed fruit (peaches and pears). These observations indicate that environment played no significant role in the possible creation of the acid tolerance of the cultures studied. This suggestion is supported by the fact that no purified cultures were carried on media with pH values less than 6.0. Yet transfers of cultures from these nearly neutral media to highly acid media grew and caused decomposition of the fermentable constituents of the media.

It is believed rather generally, with some substantiating evidence, that neither *Aerobacter aerogenes* nor *Aerobacillus macerans* is particularly tolerant of acid surroundings. From the ecological standpoint most consider these two species to be found growing only in neutral or nearly neutral surroundings such as waters and soils, low-acid vegetables, cereals, and other neutral products. (Consult Levine: Iowa State Coll. Eng. Expt. Sta., Bull. 62; Porter, McCleskey, and Levine: J. Bact., **33**, 163; Parr: Bact. Revs., **3**, 1; Vaughn and Levine: J. Bact., **44**, 487.)

The mere isolation of either *Aerobacter aerogenes* or *Aerobacillus macerans* from acid substrates does not constitute proof of their ability to tolerate and, more particularly, to grow actively in such acid surroundings as described. Since, however, these bacteria were proved to grow and decompose fermentable carbohydrates at such low pH values, it seems advisable to suggest the possibility that present conceptions of acid tolerance among certain groups of bacteria should be re-evaluated. Precedent for such re-evaluation of limits of tolerance of bacteria to acid surroundings has been set by Spiegelberg (J. Bact., **31**, 85; Food Research, **5**, 115) and Townsend (J. Bact., **36**, 315; Food Research, **4**, 231) who worked with *Clostridium pasteurianum*.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MICHIGAN BRANCH

DETROIT, MICHIGAN, NOVEMBER 16, 1945

SOME OBSERVATIONS IN THE STUDY OF FALSE POSITIVE KAHN REACTIONS. *Joseph A. Kasper.*

A number of factors may account for equivocal or false reactions. Upper respiratory infections, pregnancy, and other conditions, not yet explained, were observed to account for such reactions. This opinion is based upon the observations that when the acute infections subside, or when the pregnancies are terminated, the serological findings become negative.

From the study of 39 specimens it would seem that an increase in the fibrinogen content in the serum may account for some of the false positive findings when the Kahn standard test is employed. Positive reactions became negative after the serum had been heated at 65 C for 30 minutes. Moreover, reactions originally positive when *Salmonella* agglutinins were demonstrated in the sera became negative after a period of several weeks following recovery from an acute infection, and these agglutinins disappeared. A number of specimens in which *Brucella* agglutinins were found to be associated with positive Kahn reactions could not be attributed to syphilis.

These observations would seem to suggest that an increase of fibrinogen during acute infection, appearance of latent *Salmonella* agglutinins, probably in the course of an acute infection, persistent *Brucella* agglutinins, and other factors not fully understood, as in pregnancy, can account for some of the false positive tests for syphilis.

TESTING OF DISINFECTANTS FOR USE ON SKIN OF SHEEP. *Jack D. Tiner, Frank Thorp, Jr., and C. L. Cole,* Animal Pathology and Animal Husbandry Sections, Michigan Agricultural Experiment Station, East Lansing, Michigan.

The present study was undertaken to compare quarternary ammonium and iodine preparations for possible use in disinfecting

ovine skin. The compounds tested were as follows: tincture of iodine, U.S.P. XII, colloidal iodine, colloidal iodine with 1 per cent "naccanol" added to reduce surface tension, Lugol's iodine, U.S.P. XII, and "roccal."

The technique involved the use of skin taken from lambs just prior to autopsy. Samples possessing the natural skin flora were treated with the compounds for one-minute intervals, rinsed, and then washed in sterile distilled water. Platings with tryptose agar were made in order to calculate the number of organisms per square centimeter. The percentage of survival was determined by comparison with control samples subjected to equal volumes of sterile distilled water. The number of surviving microorganisms could be raised markedly if the rinsing was done in a neutralizing agent specific for the disinfectant.

Best disinfection was obtained with the iodine preparations. Oxalated blood counteracted to a slight degree the effect of iodine compounds. A test was also made of the relative efficiencies of some of the compounds in depressing the numbers of microorganisms composing the flora of the wool of adult sheep. Tincture of iodine was most effective for this purpose. Conclusive evidence has been obtained that aqueous roccal is precipitated by some substance on adult sheep and has no value as a disinfectant for unwashed wool.

CATALASE ACTIVITY OF *HEMOPHILUS PERTUSSIS*. *Lucile M. Portwood,* Bureau of Laboratories, Michigan Department of Health.

Quantitative determinations of catalase activity were made on 66 strains of *Hemophilus pertussis*. Rough strains showed little or no activity. There was no significant quantitative variation among the smooth strains having catalase activity. Of 62 smooth strains, 14 (23 per cent) dem-

onstrated no activity. All 62 cultures had the typical characteristics of smooth strains. Agglutination tests with adsorbed sera presented no evidence of a difference in the antigenic structure of strains with or without catalase activity. There was no correlation observed between the *absence* of catalase activity and any other property characteristic of smooth strains of *H. pertussis*.

After repeated subculture, three strains showed an alteration in properties which typify smooth strains; there was a decrease in the agglutination titer, in the virulence for mice, and in the skin-necrotizing property. Accompanying these changes was a loss in catalase activity. Strains which showed no change in these properties on repeated subculture also exhibited no variation in catalase activity. These data raise the question as to whether a loss of catalase activity by a strain of *H. pertussis* might be correlated with dissociation of that culture.

GONOCOCCUS CULTURES: COMPARISON OF RESULTS WITH MAILED SLANTS AND IMMEDIATE PLATES. *Grace Eldering and Evelyn Palser*, Bureau of Laboratories, Michigan Department of Health, Western Michigan Division.

With slants of bacto proteose no. 3 agar and hemoglobin in screw-capped tubes, specimens for gonococcus culture were sent through the mail; the exudate was spread directly on the slanted medium, without the use of broth. The time in transit from the clinic to the laboratory was approximately 24 hours. Gonococci were found in 132 of

902 mailed specimens. Of the corresponding cultures on plates, incubated within two hours, 178 were positive. There were 87 positive findings with slides. Expressed in percentage, the positive results with slides, slants, and plates were 9.6, 14.6, and 19.7, respectively. Compared with slides, the increase in positive findings with slants was 51.7 per cent, and with plates 104.6 per cent.

Further studies are in progress pointed toward improvement of the procedure and the definition of its limitations.

A GERMICIDAL FACTOR (G_{1-R}) PRESENT IN GASTRIC SECRETIONS OF CERTAIN ANIMALS INCLUDING MAN. *Ada May Ames, Wave Elaine Culver, and Walter J. Nungester*.

The accidental discovery that the gastric contents of the rat rapidly destroyed the spores of *Bacillus globigii* led to a further study of the phenomenon. A similar bactericidal activity was observed in gastric specimens from swiss mice, *Peromyscus* mice, dogs, and human beings. This factor is not lysozyme, as shown by a comparison of the properties with regard to pH, temperature, and activity against various organisms—especially *B. globigii* and *Micrococcus lysodeikticus*. The factor is not derived from salivary contamination of the stomach contents, as shown by its absence in the saliva of rats the gastric secretions of which were active. It is also active against bacteria other than *B. globigii*, including *Eberthella typhosa*, *Bacillus anthracis*, and *Salmonella enteritidis*.

NOTE ON THE PRODUCTION OF SOLUBLE BLUE PIGMENT IN SIMPLE MEDIA BY *ACTINOMYCES COELICOLOR*

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In the last paper concerned with this topic to appear in this journal (Conn, 1943; see also Conn and Conn, 1941, for relevant literature) the suggestion was made that the pigment of *Actinomyces coelicolor* was similar in chemical nature to azolitmin, the characteristic coloring matter of litmus. The fact remains, however, that no method for the preparation of the solid pigment, except in an unsatisfactory crude state, has really been described. Hence such comparisons can have but little validity at the moment. This note is mainly concerned with a description of the simplest possible medium required for the pigmented growth of the microorganism, and of a very simple method for the isolation of the true pigment from the metabolic solution.

The writer's experience is that the dark-colored precipitate thrown down by acidification of the usual purple metabolic solution (Conn, 1943) is never even a satisfactory crude preparation of the true bacterial pigment. Many preliminary experiments showed that the product was always contaminated with much acid-insoluble protein if the medium initially contained a fermentable sugar (or sugar alcohol) or an amino acid. Attention was therefore concentrated on the ammonium salts of the simpler, naturally occurring nonnitrogenous acids, readily available in a pure state, as sole sources of both carbon and nitrogen. Of these, only ammonium acetate proved worthy of consideration, the microorganism refusing to grow on formate, and yielding only yellow metabolic solutions on succinate, fumarate, or citrate.

EXPERIMENTAL

The medium finally chosen for pigment production was prepared as follows: A basal inorganic salt solution was made of the following composition (g per l)— K_2HPO_4 , 4.8; NaCl, 1.2; $MgSO_4 \cdot 7H_2O$, 0.08; $FeSO_4 \cdot 7H_2O$, 0.005. This liquid was distributed in 100-ml lots in half-liter conical flasks, which were plugged and sterilized by autoclaving in the usual way. A solution of glacial acetic acid (70 ml) in distilled water (200 ml) was brought to pH 7 by cautious addition of concentrated ammonia solution and finally made up to 1 liter. It was sterilized by steaming, and 10-ml quantities were added aseptically to the flasks containing the sterile basal medium. The inoculum was prepared from slopes 1 to 3 weeks old (which had been incubated at 24 C after streaking with a little of a sporing growth of *A. coelicolor*), of the following composition (g per L): sucrose, 10; $NaNO_3$, 2; K_2HPO_4 , 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; agar, 20. The thin but plentifully sporing growth so obtained was rubbed up lightly with a few ml of sterile water, and each slope was used to inoculate one, or at most two,

flasks, which were then incubated at 24 C for at least a month. A poor surface felt developed in a week or 10 days, but the purple color did not appear in the metabolic solution until about 2 weeks had elapsed. At the end, the metabolic solution was filtered in the ordinary way through a fluted filter paper, and the filtrate¹ was acidified with 0.1 volume of 2 N HCl and left overnight for settlement of the precipitate. The clear supernatant was decanted as far as possible, and the residue collected and washed twice with water on the centrifuge. It was then dried in a vacuum desiccator containing both solid KOH and concentrated H₂SO₄. This crude product (finely powdered) was repeatedly treated with 100 parts of cold glacial acetic acid, and the soluble part, always less than half of the whole, was recovered by dilution of the filtered red solution with much water. The precipitate, which still contained protein (Found: N, 2.4 per cent) was collected as before, dried, and triturated with cold redistilled acetone. The true pigment, although still amorphous, was obtained by evaporation of the filtered acetone extract at room temperature. The quantitative aspect of these

TABLE 1
A. coelicolor on 0.7 per cent ammonium acetate

STRAIN OF <i>A. COELICOLOR</i> *	PERIOD OF INCUBATION	VOL. OF FIL- TERED META- BOLIC SOLUTION	WT. OF CRUDE ACID PRECIPITATE	WT. OF AcOH SOLUBLE PART	YIELD OF ACETONE-SOLU- BLE PIGMENT
	days	L	g	g	mg
BCA1.....	32	6	0.5	0.1	30
U 1010.....	40	3.5	0.12	0.01	trace
BCA1.....	35	2.6	0.12		ca3

* BCA1 was a recent isolate from soil, and U 1010 an old stock culture.

I am indebted for both these cultures to Dr. H. G. Thornton, F.R.S., and Mrs. D. H. Oxford of the Department of Soil Microbiology, Rothamsted Experimental Station.

experiments is summarized in table 1. The pigment cannot be extracted by acetone from the crude acid precipitate.

Properties of the acetone-soluble pigment. The best preparation still contained 1.9 per cent N, probably due to a residual protein contamination. Its further purification will be described in another place, but four of its distinctive properties may be mentioned here:

(1) Its deep blue solution in N NaOH is relatively stable but definitely fades somewhat and takes on a greener hue when left overnight at room temperature.

(2) It yields very similar shades of purple by solution in 0.1 per cent K₂HPO₄ solution, or in concentrated H₂SO₄.

(3) It seems to be quite insoluble in hydrocarbon solvents and hardly soluble at all in any of the ordinary neutral organic solvents with the exception of acetone and the simple alcohols.

(4) Addition of 0.1 N alcoholic potash to its red alcoholic solution yields a blue precipitate which settles to reveal an absolutely colorless supernatant.

¹ Generally speaking such filtrates are suitable for the isolation of pigment only if the deep blue color produced on addition of 0.1 volume of 10 N NaOH does not fade or become green on standing for several hours.

DISCUSSION

It may be concluded, even at this preliminary stage, that the pigment contains too little nitrogen to substantiate either the azolitmin suggestion of Conn (1943) or the phenazine suggestion of Erikson *et al.* (1938). The remarks of the last-named authors concerning the invalidity of the anthocyanidin structure are, however, still pertinent. It is evident that most preconceived ideas as to the chemical nature of this remarkable pigment are of little relevance, and the whole problem will have to be reinvestigated *ab initio*. Furthermore, the author is of the opinion that it is absolutely necessary to isolate the true pigment, by some such procedure as here outlined, before the tentative conclusion of Conn (1943) that the pigment of *A. violaceus-ruber* is different from that of *A. coelicolor* can be accepted; for in the crude state the true acetone-soluble pigment is always accompanied by protein, which greatly modifies its characteristic color reactions.

SUMMARY

A. coelicolor has been grown on a medium containing ammonium acetate as the sole source of carbon and nitrogen, and the true acetone-soluble pigment, responsible for the characteristic color of this organism, has been isolated from the metabolic solution. The pigment is probably not an anthocyanidin, nor a phenazine, nor related to azolitmin; in fact, nothing is really known about its chemical nature.

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THE ANAEROBIC BACTERIAL FLORA OF CLOSTRIDIAL MYOSITIS

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During the first World War, several workers investigated the bacterial flora of wounds of patients suffering from gas gangrene. The most extensive work was that reported by Weinberg and Seguin (1918) and by a group of British workers (Report, 1919). Since that time, there has been no report of the investigation of any large number of cases until MacLennan (1943) reported on his findings in battle casualties of the British Eighth Army. The majority of the cases on which he reported resulted from the fighting which took place on the North African desert. Stock (1944) reported on the bacteriological findings in 25 cases of gas gangrene in Italy, the chief object of his study being to determine the incidence of *Clostridium novyi*.

The present paper deals with the incidence of anaerobic bacteria in 110 cases of clostridial myositis in battle casualties of the Fifth Army. Most of the cases were casualties from the fighting between the Volturno and the Garigliano rivers, the crossing of the Rapido, and from Cassino, although some were received from the beach head at Anzio. The work was done in a mobile laboratory unit of the 15th Medical General Laboratory and in the Bacteriology Section of the main installation of this unit.

MATERIAL AND METHODS

During the greater part of this investigation Brewer jars were used to provide anaerobic conditions for the incubation of nutrient agar plates. During the first 5 months, however, these were not available, and anaerobic jars were improvised from 105-mm howitzer shell cases, following a pattern shown to us by Major A. H. Stock, M. C. A brass collar was brazed to the open end of the shell case, and an iron disc and the collar were ground to fit. Modeling clay or a rubber gasket cut from an inner tube was used as a seal between the iron lid and the collar. Through the center of the lid, a tightly fitting one-hole rubber stopper and a glass stopcock provided for the evacuation and the filling of the jars with hydrogen. Platinized asbestos was used as a catalyst.

Blood agar plates were prepared from a base of dehydrated heart infusion broth (Difco) to which 5 per cent defibrinated human blood was added. When Kahn-positive citrated blood from a blood bank was used, sufficient calcium chloride was added to the blood agar base to combine with the citrate and to furnish a slight excess of calcium. If this were not done, the hemolysis patterns of several species were atypical.

For routine transfers, a stock semisolid medium was used. This consisted of brain and heart infusion broth, to which was added 1 per cent tryptone and

¹ Captain, Sn.C.

0.15 per cent agar. The same medium also served satisfactorily for the determination of indole production. Dimethylaminobenzaldehyde in amyl alcohol and hydrochloric acid was used to test for this substance. Strips of filter paper soaked in a saturated solution of oxalic acid were not so sensitive for the determination of indole production as was the dimethylaminobenzaldehyde indicator.

The semisolid medium used as a fermentation base and the medium used to test for the reduction of nitrate to nitrite were those described by Reed and Orr (1941). However, no sodium thioglycollate was used, since comparison of the results of a large series indicated not only that the use of this substance was no aid but that, in certain cases, it appeared to be slightly inhibitory. The iron-milk and iron-gelatin media were those of Spray (1936). The medium used for the determination of the liquefaction of coagulated egg albumen was not the usual one, owing to our inability to procure fresh eggs in the field. However, Major William H. Ewing, Sn. C., of the 15th Medical General Laboratory, devised a medium which appeared to be superior in several respects to the one usually used. This was an 8.5 per cent suspension of whole dried egg in brain and heart infusion broth. Autoclaving served to sterilize the medium and to coagulate the egg albumen in a semisolid mass. Digestion was readily apparent, and all species, proteolytic or not, appeared to grow well in the liquid medium held in the interstices of the coagulated albumen.

The sugars used for fermentation were prepared in 10 per cent solution in distilled water and were sterilized by filtration through Seitz filters. The sterile sugar solutions were added to the freshly heated semisolid fermentation media to give a final concentration of 1 per cent sugar.

Tubes of differential media were inoculated with 0.1 to 0.2 ml of overnight cultures in the stock semisolid medium. The inoculation was made by means of 1-ml pipettes, the inoculum being placed at the bottom of the tubes of differential media. Incubation was at 37 C. All tubes were examined daily for 5 to 7 days; fermentation of the sugars was tested by removing, with sterile precautions, a drop of the culture and adding to it a drop of bromthymol blue indicator on a spot plate.

The following were used for cultural identification in addition to the colony, hemolytic, and morphological characteristics: fermentation of glucose, maltose, lactose, sucrose, and salicin; liquefaction of gelatin, casein, coagulated egg, and coagulated serum; reduction of nitrate to nitrite; production of indole; reaction in iron-milk medium; production of hydrogen sulfide; blackening of iron-brain medium.

The specimens taken for culture were portions of muscle from the affected area. These specimens were taken with care from muscle which was grossly infected and were never adjacent to the surface of the wound. This was done to avoid bacteria which might be present on the wound surface and which were not involved in the developing infection of the muscle. The specimens were placed in screw-capped vials or chopped meat medium and taken to the laboratory for culture. Blood cultures were occasionally done, but the results are not considered in this report.

The procedure for isolation was as follows: The specimen was streaked on three blood agar plates, two of which were incubated anaerobically and one aerobically. A tube of iron-milk was usually inoculated at the same time; this was subcultured to anaerobic plates after 24 hours' incubation. Anaerobic plates were allowed to incubate for 3 days, when they were examined. Organisms of each of the various colony types were examined microscopically and inoculated into semisolid stock medium; after 24 hours' incubation, these were again streaked on blood agar plates and incubated for 3 days. If the second plating was pure, a typical colony was transferred to semisolid stock medium. If the growth in this medium was satisfactory, the culture was tested for the presence of aerobic contaminants and for microaerophilic tendencies by streaking an aerobic blood plate and examining this after 24 and 48 hours of incubation. Anaerobic organisms were then transferred to semisolid stock medium, and this culture, after overnight incubation, was used to inoculate the differential media. After the differential media had been incubated for 5 to 7 days, anaerobic plates were inoculated and incubated as a further test of purity of the strain.

Although but two platings are described in the preceding paragraph before inoculation into differential media, five or six platings were sometimes necessary to be certain that all contaminants had been eliminated. Difficulties with organisms showing swarming tendencies were partially overcome by increasing the concentration of agar in the blood agar base to 4 to 6 per cent. With this increased concentration of agar, there was little swarming, but the colony characteristics, and the extent of hemolysis produced, were markedly changed.

Specimens from which no pathogenic anaerobic organisms were isolated at the first or second plating were passed through enrichment media and replated. Three passages through milk, the lactose of which served to increase the relative numbers of *C. perfringens*, were made, and the culture was then plated from the last milk tube. It was then passed three times through glucose gelatin, which served to increase the relative number of *C. novyi*. No pathogenic anaerobic bacteria were found in some specimens, even after enrichment passage. One-half ml of the supernatant fluid of the chopped meat tubes of such specimens was injected into guinea pigs. If the animals died, isolation was made from the site of injection and from the heart's blood.

All cultures which were classified as belonging to pathogenic species and all cultures designated "unidentified" were tested in guinea pigs. The animals were inoculated with 0.3 ml of an 18-hour culture mixed with an equal quantity of sterile 5 per cent calcium chloride, or with 0.5 ml of an 18-hour broth culture. Injections were made into the muscle of the thigh. The animals which received monovalent antitoxic sera were given this intraperitoneally, in doses of 50 to 75 units 3 hours before the injection of the culture. Specific monovalent antisera were obtained from Major J. D. MacLennan, R. A. M. C., of the British Eighth Army, from Dr. Douglas McClean of the Lister Institute, and from Miss Nancy Hayward of the Ministry of Health Emergency Service. Miss Hayward also furnished us with specific antihemolytic sera.

FINDINGS

The anaerobic species of bacteria found in 110 cases of clostridial myositis, and the percentage of cases in which they occurred, are given in table 1. The species found most often were *Clostridium sporogenes* and *Clostridium bifermentans*, both proteolytic species whose role in infections of this type is not known. Stock, also working in Italy, found a high incidence of these two organisms, although we found more *C. bifermentans* and fewer *C. sporogenes* than he reported. This difference may arise from the fact that we did not attempt to differentiate between *C. bifermentans* and *Clostridium centrosporogenes*.

TABLE 1

Anaerobic organisms found in specimens from 110 cases diagnosed as clostridial myositis

SPECIES	NUMBER	PER CENT
<i>C. sporogenes</i>	60	54
<i>C. bifermentans</i>	60	54
<i>C. perfringens</i> (<i>B. welchii</i>).....	43	39
<i>C. novyi</i> (<i>B. oedematiens</i>)	35	32
Anaerobic cocci	25	23
<i>C. multifermentans</i>	5	5
<i>C. tetani</i>	4	4
<i>C. capitovalis</i>	3	3
<i>C. butyricum</i>	3	3
<i>C. fallax</i>	3	3
<i>C. tertium</i>	3	3
<i>C. cochlearium</i>	2	2
<i>C. putrificum</i>	2	2
<i>C. regulare</i>	2	2
<i>C. sphenoides</i>	2	2
<i>C. paraputrificum</i>	1	1
<i>Fusobacterium</i> sp.....	1	1
Unidentified*	58	

Mean number of anaerobic species per case—2.84.

* Not agreeing with the description of any species listed in *Bergey's Manual of Determinative Bacteriology*.

C. sporogenes was considered by the British workers during the World War to hasten and aggravate infections caused primarily by *Clostridium perfringens*, *Clostridium septicum*, and *Clostridium novyi*. Although the strains isolated by us were not pathogenic for guinea pigs, except in rare instances, it appeared that *C. sporogenes* was not entirely avirulent for man, for several cases were encountered in which muscle groups were widely invaded by *C. sporogenes* in apparently pure culture. Mild toxemia was evident in these patients, but the invasion of muscle did not extend beyond the muscle groups which had impaired blood supply.

Of the strains of *C. bifermentans* isolated by us and tested in guinea pigs for pathogenicity, only about 5 per cent were sufficiently virulent to cause the death of the animals. About one third of the strains produced local necrosis, which healed spontaneously. When the injection of the culture was preceded by an

injection of 5 per cent calcium chloride, about 10 per cent of the strains caused complete digestion of the skin and muscle of the limb involved, resembling the digestion brought about by *Clostridium histolyticum*. Although digestion proceeded so rapidly that only the bones of the leg remained after 48 hours, the animals did not die; generally the infection was limited to the hind leg. Such animals showed no evidence of the toxemia which one might expect to result from the absorption of the products of proteolytic digestion; they exhibited smooth coats, continued to eat and drink, and moved about as well as might be expected. There appeared to be some loss of virulence in strains of *C. bifermentans* cultured in chopped meat media, for several strains which once had killed guinea pigs within 24 hours were apparently without effect when tested again several months later. The colonial and cultural characteristics had not changed during this time.

The most commonly found toxigenic species was *Clostridium perfringens* (*B. welchii*), which occurred in 43 of 110 cases of clostridial myositis. All the strains of this organism were found to be virulent for guinea pigs, and most of them exhibited a double zone of hemolysis on blood agar plates. A few strains with colonies resembling those of *Staphylococcus albus* were isolated from chest wounds which had been treated with penicillin. These strains were pathogenic for guinea pigs, and when isolated after animal passage showed colonies typical of *C. perfringens*, surrounded by double zones of hemolysis.

Clostridium novyi (*B. oedematiens*) was isolated from specimens from 35 of the 110 cases of clostridial myositis. More trouble was experienced with this species than with any other in isolation in pure culture. Its almost constant tendency toward swarming on blood agar plates containing 2 per cent agar made the picking of isolated colonies impossible until the agar concentration was raised to 4 to 6 per cent. Picking from the edge of the swarming area generally did not yield pure cultures, for *C. sporogenes* appeared to swarm almost equally well in the presence of such a strain. Furthermore, the blood agar base available to us was not entirely suitable for the cultivation of *C. novyi*, for a number of strains refused to grow on this medium in pure culture. Others would grow only when heavy inocula had been used; isolated colonies were not found. The addition of 1 per cent tryptone improved the medium somewhat, but it was found that this did not compare with infusion agar prepared from fresh meat. Unfortunately, fresh meat was not always available.

The virulence of *C. novyi* for guinea pigs was relatively constant. Only one strain was encountered which was not highly virulent, and this strain produced death of the animals if its injection were preceded by an injection of 5 per cent calcium chloride. The production of hemolysin on blood agar plates was usually not marked, being much less than that produced by *C. perfringens* or by many strains of *C. bifermentans*, and resembled that produced by many strains of *C. sporogenes*. This was probably a characteristic associated in some fashion with the inadequacy of the medium, for the production of hemolysin in semisolid stock medium was usually excellent, either in the presence or the absence of sodium thioglycollate.

Anaerobic cocci were isolated from specimens from 25 of the 110 cases. Cultural characteristics of ten of these strains were determined, but species identification was not made, since none of them were in agreement with the species described in *Bergey's Manual of Determinative Bacteriology* or in *La Gangrene gazeuse* of Weinberg and Seguin (1918). Furthermore, the characteristics of these strains seemed to be changing during their existence on laboratory media.

The other species listed in table 1 were found in only a few of the specimens. The four strains of *Clostridium tetani* were fully toxigenic for guinea pigs. These strains were all isolated from wounded American soldiers. Apparently, immunization with toxoid does not prevent the growth of this species, although no symptoms of tetanus were present. The immunity produced appears to be purely antitoxic. The pathogenicity of the strains designated as *Clostridium fallax* could not be tested until some months after their isolation. At this time they were found to be nonvirulent for guinea pigs.

The strains designated "unidentified" exhibited cultural, colony, or morphological characteristics which did not allow us to consider them identical with any species listed in *Bergey's Manual of Determinative Bacteriology*. These strains, for the most part, were avirulent for guinea pigs, only a few being sufficiently pathogenic to cause death.

In this series of cases we did not encounter any strains of *C. septicum*, a toxigenic organism which was often found by the investigators during the World War. The adequacy of the procedures used by us for isolation was checked by inoculating specimens with known cultures of *C. septicum* and then culturing in the usual fashion. Apparently the methods were adequate. After the present survey was completed and the casualties came from the fighting in the Apennines, *C. septicum* was occasionally encountered. Stock found only 1 strain of *C. septicum* in specimens from 25 cases while the fighting was in southern Italy, but encountered it more frequently the following year.

The number of different species given in table 1 shows how widely the flora of anaerobic infections will vary. That these infections are not always due to one toxigenic species is illustrated by the fact that in 63 cases in which *C. perfringens*, *C. novyi*, or both, were found, the former alone occurred in 28 cases, the latter alone in 20, whereas both of them were found in the specimens from 15 cases. The mean number of anaerobic species found per case was 2.84, a result slightly higher, though not significantly so, than that of MacLennan, 2.62, or that of Stock, 2.56. In the majority of the cases more than two anaerobes were found. Whether the less toxigenic species aid or hasten the invasion of tissue by the more toxigenic species is not definitely known; certainly this problem deserves more attention than has been devoted to it thus far.

In a number of cases, two or more specimens were taken and handled separately in the bacteriological examination. With these, as with all specimens in this investigation, care was taken to obtain material at some distance from the wound surface in order to avoid contamination that might be present on the surface of the wound. This procedure was based on the assumption that only the more

TABLE 2
Anaerobic organisms found in multiple specimens

Specimen 1A <i>C. sporogenes</i> <i>C. perfringens</i> <i>C. sphenoides</i>	Specimen 1B <i>C. sporogenes</i> <i>C. bifermentans</i> Unidentified <i>Clostridium</i>	
Specimen 18A <i>C. bifermentans</i> <i>C. tertium</i>	Specimen 18B <i>C. bifermentans</i> <i>C. sporogenes</i>	Specimen 18C <i>C. sporogenes</i> <i>C. bifermentans</i>
Specimen 19A <i>C. novyi</i>	Specimen 19B <i>C. perfringens</i> <i>C. novyi</i>	Specimen 19C <i>C. bifermentans</i>
Specimen 28A <i>C. perfringens</i> <i>C. sporogenes</i> Unidentified <i>Clostridium</i> Specimen 28D <i>C. sporogenes</i> Unidentified <i>Clostridium</i>	Specimen 28B <i>C. sporogenes</i> Unidentified <i>Clostridium</i> Specimen 28E <i>C. sporogenes</i> <i>C. capillovale</i> <i>C. novyi</i>	
Specimen 29A <i>C. perfringens</i> <i>C. bifermentans</i> Two unidentified clostridia	Specimen 29B <i>C. novyi</i> <i>C. bifermentans</i>	
Specimen 36A <i>C. novyi</i>	Specimen 36B <i>C. novyi</i>	
Specimen 44A <i>C. perfringens</i> <i>C. sporogenes</i>	Specimen 44B <i>C. perfringens</i> <i>C. sporogenes</i> <i>C. bifermentans</i> Unidentified <i>Clostridium</i>	
Specimen 45A <i>C. sporogenes</i> Unidentified <i>Clostridium</i>	Specimen 45B <i>C. perfringens</i> <i>C. sporogenes</i>	
Specimen 52B <i>C. novyi</i>	Specimen 52D <i>C. tetani</i>	
Specimen 60A <i>C. perfringens</i> <i>C. bifermentans</i>	Specimen 60B <i>C. perfringens</i> <i>C. bifermentans</i>	
Specimen 60XA <i>C. bifermentans</i>	Specimen 60XB <i>C. bifermentans</i> <i>C. sporogenes</i>	
Specimen 97A <i>C. sporogenes</i>	Specimen 97B <i>C. novyi</i>	Specimen 97C <i>C. novyi</i>
Specimen 101A Anaerobic cocci	Specimen 101B <i>C. novyi</i> Unidentified	

pathogenic species could invade tissue, and that secondary invaders might be avoided by this means. The results of the investigation of multiple specimens, shown in table 2, indicate that this is not entirely correct and that pathoge-

nicity—which is closely associated in this group of organisms with exotoxin production—has little to do with ability to invade muscle, especially muscle which has been partially or completely devitalized by impaired blood supply, trauma, or bacterial toxins. Further, it is evident that the invading bacteria were not uniformly present throughout the affected tissue and that the uniformity of spread varied with different species. *C. perfringens*, for example, was found in 7 of these cases; only twice was it found in both specimens; in the other 5 cases it was found in only 1 of 2 or 3 specimens. *C. sporogenes*, on the contrary, was found in 7 cases; in only 2 of these was it found in 1 specimen. *C. novyi* and *C. bifermentans* were between *C. perfringens* and *C. sporogenes* in this regard. It is interesting to note that *C. perfringens*, the least uniformly distributed of these species, is the only nonmotile one.

Although a relatively small number of cases were treated in this fashion, it is evident that the bacteriological examination of a single specimen of invaded muscle will often be insufficient to demonstrate the presence of all anaerobic bacteria invading the tissue. It is equally evident that there is little relation

TABLE 3
Comparison of distribution of anaerobes in fatal and nonfatal cases

TYPE OF ANAEROBE	FATAL CASES		NONFATAL CASES	
	Number	%	Number	%
<i>C. sporogenes</i>	16	61	17	40
<i>C. bifermentans</i>	14	54	22	48
<i>C. perfringens</i>	8	31	21	46
<i>C. novyi</i>	9	35	15	33
Anaerobic cocci.....	6	23	12	26
Total	26		43	

between toxigenicity and invasiveness once an infection is set up. The part played by the less toxigenic but more invasive bacteria, such as *C. sporogenes* and *C. bifermentans*, is not known. Definite knowledge on this problem of bacterial synergism is needed.

A comparison of the frequency with which the five species of anaerobic bacteria most commonly encountered in this series were found among the fatal as compared with the nonfatal cases is shown in table 3. Since all these cases were treated by surgery, and for the most part with antitoxin, the distribution of any species may be taken to reflect, to some degree, the adequacy of the treatment in overcoming the harmful effects of the organism in question. In this regard, it should be pointed out that although the antitoxin of American manufacture was prepared primarily against the toxins of *C. perfringens* and *C. septicum*, with little antitoxin against *C. novyi*, this lack was compensated for by the use of antitoxin of British or Australian manufacture, when it could be procured.

It will be noted that *C. sporogenes* was found to be present in a higher percent-

age of fatal than nonfatal cases. Although the number of cases in each group is small, the probability that a difference of this size might occur by chance is only 1 in 12. This may indicate that *C. sporogenes* does not play an entirely innocuous role in these mixed infections, although the data are not sufficient to warrant conclusions. However, the relatively high incidence of this species in fatal cases and the uniformity with which it invades muscle which has been partially devitalized by trauma or impaired blood supply warrant further investigation, with the possibility that it may have a synergistic action in mixed infections.

SUMMARY

The spore-bearing anaerobes most often encountered in clostridial myositis in Italy were *Clostridium sporogenes*, *Clostridium bifermentans*, *Clostridium perfringens*, and *Clostridium novyi*.

The organisms were not evenly distributed throughout the affected tissue. *C. sporogenes* was most widely distributed; *C. perfringens* had the most haphazard distribution.

It is probable that the progress of mixed anaerobic infections is actively aided by *C. sporogenes*.

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THE OCCURRENCE OF TYPE 10 PARACOLON IN TURKEYS

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Peluffo, Edwards, and Bruner (1942), Edwards, Cherry, and Bruner (1943), and Edwards (1945) have discussed coliform bacteria that possess antigenic components similar to those found in the genus *Salmonella*. In the second paper they described a "type 10" which was originally found in turkey poults and in a captive rattlesnake in the same section of California in 1942. The antigenic formula given by them is XVIII:z₄, x₂, x₇. It is the purpose of this paper to report the occurrence of this type in turkeys with reference to its importance as a cause of mortality. In a previous paper the writers (1944) described the occurrence of type 8 paracolon (GH:z₄, z₂₃, z₂₆) as a cause of mortality in turkeys and reported its presence in snakes.

HISTORY OF OUTBREAKS

Type 10 paracolon was first recognized as a probable cause of mortality in poults in one area of California in 1943. It was isolated from poults from two consecutive hatches 1 week apart. From the hatch of June 17 it was recovered from 3 of the 5 ranches receiving poults. From the hatch of June 24 it was recovered from 1 of 5 ranches receiving poults. In addition to these isolations we isolated it from 6 apparently normal adult birds killed on 3 other ranches in 1943, making a total of 7 infected ranches in 1943.

In 1944 it was found in poults in 6 outbreaks on 5 ranches, and in 3 adult carriers from 2 ranches. One of these ranches is 1 of the 5 mentioned above. No diagnosis was made in poults from the second ranch. Thus, at least 6 ranches were involved in 1944. The poults in 5 of the outbreaks were received from the same hatchery, which is the one also concerned in the 1943 outbreaks. The sixth one occurred in birds hatched in another area, but from eggs purchased from the above-mentioned region.

The mortality in the 5 outbreaks in 1943 ranged from 10 to 70 per cent. On the ranch which suffered a 70 per cent mortality the greatest loss occurred during the first 3 weeks, but losses continued for 5 weeks. Three lots of poults were obtained for autopsy during that time (at 1, 2, and 4 weeks), and this organism was the only significant type isolated. In 1944 the losses ranged from 5 to 35 per cent. In the lot suffering only a 5 per cent mortality, the morbidity was high. In the 1944 outbreaks it was possible to trace 4 of the groups of poults to a common egg source. It is also interesting to note that these eggs came from the ranch which had the low mortality, but heavy morbidity, early in the season.

In 1945 there were no outbreaks in poults reared by the members of the co-operative group where previous outbreaks had occurred. This is probably due to the fact that no eggs from any 1944 infected flocks were used for 1945 replace-

ments. Losses from type 10 infection were, however, experienced by 4 turkey growers (5 outbreaks) who secured poults from another hatchery located in the same area and from a hatchery located in a different area which purchased eggs from the infected area. In one outbreak studied in 1945 the losses started when the poults were 6 days old, and the mortality in 3 weeks was approximately 1,700 of the 3,000 poults hatched.

Every outbreak studied by us to date could be traced to eggs or poults originating in the area where the original cultures were isolated.

CULTURAL, ANTIGENIC, AND PATHOGENICITY STUDIES

The paracolon types isolated from the cases reported herein have been shown by cultural and antigenic studies to be identical to Edwards, Cherry, and Bruner's type 10 paracolon. In the 3 years a total of 63 cultures isolated from 19 outbreaks on 17 turkey ranches have been studied.

Cultural studies. Detailed cultural studies were made on nine strains (Pc 52, 53, 55, 56, 72, 73, 74, 75, 76) isolated from poults and were compared with one known type 10 strain (Pc 27). All gave similar reactions. They were motile, gram-negative rods, which were methyl-red-positive and Voges-Proskauer-negative. Abundant hydrogen sulfide was produced from proteose-peptone ferric citrate agar and from cysteine gelatin in 24 to 48 hours. Gelatin was liquefied slowly by all cultures, liquefaction being complete in 7 cases in 3 weeks and in 2 cases in 1 month (72 hours' incubation at 37 C and then at room temperature). All of them were indole-negative, gave no reaction in Jordan and Harmon's *d*-tartrate agar, and grew poorly in Koser's citrate broth; and in potassium nitrate broth nitrate was reduced to nitrite (tested after 72 hours). Growth on potassium nitrate agar gave a good positive reaction for nitrate reduction after 72 hours' incubation. Litmus milk became slightly acid in all cases in 24 hours. Six cultures returned to neutral and remained so for at least 3 weeks; 2 became neutral in 1 week and returned to alkaline in 3 weeks; 1 returned to neutral in 1 week, became alkaline at 2 weeks, and at 3 weeks had formed a rennet curd (2 trials). Growth in tetrathionate broth (Difco plus 1:50,000 brilliant green) was good. On 1:25,000 brilliant-green meat extract agar, desoxycholate agar (B.B.L.) both with and without citrate, Difco SS agar, and Kauffman's neutral-red brilliant-green agar, the cultures resembled *Salmonella* in rate of growth and type of colonies.

Lactose was not fermented in 3 weeks on original isolation, and when retested after storage for several weeks in cotton-stoppered tubes, only two cultures showed acid production with no gas in 60 days. Although lactose was either not fermented at all or very slightly in 60 days (2 trials), the majority of the cultures seeded on desoxycholate agar showed many lactose-positive daughter colonies after 3 weeks (72 hours at 37 C, then at room temperature). Lactose-positive papillae from two such plates (Pc 72, Pc 73), seeded into 1 per cent lactose fermentation tubes, produced acid and a small amount of gas in 72 hours. Subcultures from these lactose tubes again produced acid and a small amount of gas in 72 hours. These studies demonstrate the ability of this type to ferment lactose under proper environmental conditions.

A summary of the other carbohydrate and alcohol reactions observed over a period of 3 weeks is as follows: Acid and gas were produced within 48 hours in glucose, maltose, rhamnose, xylose, arabinose, sorbitol, mannitol, and trehalose. No acid or gas was produced in sucrose, dulcitol, salicin, inositol, and cellobiose. Glycerol was slowly fermented, and by the end of 1 month all cultures showed an acid reaction, but none had produced any gas. Growth in Bitter's medium containing maltose, rhamnose, and xylose was good. In 24 hours Bitter's maltose and xylose gave a strong methyl red test; all rhamnose cultures were weakly positive.

Antigenic studies. The O and H antigens of all the strains were agglutinated by the Edwards, Cherry, and Bruner type 10 antiserum. These results were verified by Dr. Edwards with representative strains sent to him. To determine whether the cultures were antigenically identical to type 10, two strains (Pc 52 and 74) were used to absorb type 10 (184) antiserum. It was possible to remove completely both the O and H agglutinins from this serum with each of the cultures. Reciprocal absorptions, using two known type 10 cultures (184, 27) were also made, and all the agglutinins were removed by their antigens. The absorbed serums were all negative at a titer equivalent to 1:100 of the original serums. Therefore, it was concluded that the cultures were identical to the known strains of type 10 previously studied by Edwards, Cherry, and Bruner (1943).

The survivors of the five 1943 outbreaks were tested for paracolon type 10 carriers during the breeding season with the following results: A total of 2,344 tests were set with the flagellar (H type) antigen, and 10 reactors on 3 ranches were found. A total of 1,761 tests were set with the somatic (O type) antigen, and 2 reactors were found on 1 ranch. The end titers of the H type reactors were 1:20, 3; 1:40, 2; 1:80, 3; 1:160, 1; 1:640, 1. One of the O type reactors had an end titer of 1:20 and the other 1:40. A total of 8,416 tests were conducted on other birds in the same area on 23 ranches, and 3 reactors on 3 ranches were found. In 1944-45, 2,021 birds were tested on 5 ranches and 3 reactors were found. Of these, 2 were in one flock and reacted only at 1:20 to the somatic antigen. The third bird reacted at 1:20 to the flagellar antigen.

Pathogenicity. Six 2-day-old Bronze poults and five 1-day-old White Leghorn chicks were each given 0.5 ml of a 24-hour broth culture of Pc 52, one of the strains referred to above, by inserting a pipette into the crop. A drop of the culture was also dropped into one eye of each individual. The poults and the chicks were brooded together. A second group of 5 poults and 5 chicks was treated in a similar manner with Pc 27, the type culture.

Five of the six poults fed Pc 52 died within 1 week, and cultures identical to Pc 52 were isolated from all of these. From three of these, all of which died within 96 hours, the organism was isolated only from the livers; from one which died on the seventh day it was isolated from the liver and intestine; and from the fifth one the organism was isolated only from the intestine. Other tissues were not cultured. One of the five chicks died in 92 hours, but there is no record of a bacteriological examination. The others were still alive in 3 weeks, but they were poor, and three of them had pasted vents, indicating that they had had an

enteritis. From two of these, the culture was reisolated from both the livers and intestines.

Four of the five poult given Pc 27 died within 10 days, the first death occurring on the fifth day. Records of bacteriological examination are available on three of the four, and the culture was reisolated from all three of these. It was recovered from the livers and intestines of all three and from the heart blood of the only one tried. None of the chicks died, but two of the five were poor and had pasted vents when killed after 3 weeks. From one of these the culture was recovered from the liver, which was enlarged.

Catarrhal enteritis was a consistent finding in the infected poult. The livers were slightly swollen and were usually congested. In some of the poult and chicks, impaction of the rectums with urates was observed. No eye lesions were noted.

Control chicks and poult which were held in separate brooders and not given the cultures did not develop the infection and were negative on autopsy.

SYMPTOMS, AUTOPSY FINDINGS, AND TREATMENT

There were no specific symptoms of this disease. The birds were listless; diarrhea was evident; pasting of the down around the vent was common; and in one outbreak acute nervousness with torticollis was common. The organism has been consistently isolated from the heart blood, livers, lungs, kidneys, unabsorbed yolk, and intestines of the poult, indicating a definite septicemia. In some cases a marked congestion of the duodenum was noted, and the livers were frequently ochre or mottled. Eye lesions, common in some types of paracolon infections, were not observed personally by us. During the spring of 1945, however, one culture sent to our laboratory for identification by Dr. M. L. Miner of Utah State College was isolated by him from the eye of a blind poult during an outbreak where eye infections were noted.

From the adult birds it was recovered from the ovary, spleen, crop, and unabsorbed yolk mass of one; from the crop of another; from the crop and intestines of two others; from the ovary, kidneys, mesentery, and intestine of another; and from the intestines of two others. Two of the birds had small caseous mesenteric lesions, and three had cystic ovules.

Preliminary experiments indicate that this type responds to treatment with certain of the sulfa drugs. At 8 days of age, one lot of 652 poult had suffered a 12 per cent mortality. The birds were in two brooders; sulfamerazine¹ was administered to one group at a dosage of 0.25 per cent in the mash for 3 days. The birds showed marked improvement as compared to those of the untreated pen. When treatment was stopped, the losses started again. Sulfamerazine treatment was repeated for 3 days to this group and was also given for 3 days to the group left as controls in the previous trial. Again the birds appeared to respond to treatment. When the survivors were tested 6 months later, no reactors to the agglutination test were found.

¹ The sulfamerazine used in this experiment was obtained through the courtesy of Sharpe and Dohme, Inc., Glenolden, Pa.

DISCUSSION

This paper reports the isolation of type 10 paracolon (Edwards *et al.*, 1943) from 15 outbreaks in turkey poults and from adult turkeys on 4 additional ranches. Type 10 was originally isolated almost simultaneously from a captive rattlesnake in a zoo, by the writers, and from poults in the same area at another laboratory. Cultures from both sources were sent to Dr. Edwards who studied them antigenically and reported them as "type 10." The mortality records in the field outbreaks and the results of laboratory-produced infections indicate that this type is definitely pathogenic for turkeys. There is also evidence (unpublished) that it is of pathogenic significance in reptiles and in certain rodents. It is evident that it can be transmitted through the egg and spread in the hatchery in the same manner as the *Salmonellae*. It is, therefore, of considerable economic importance to the turkey industry.

From the small percentage of reactors found by the agglutination test, it would appear that the infection is more easily aborted than is, for example, pullorum disease. However, as with many *Salmonellae*, it seems probable that adult birds frequently contract the infection from other animal carriers, and that they subsequently transmit the infection to poults through eggs laid by them.

From an epidemiological standpoint, it is of interest to note that all of the outbreaks reported have been confined to one area approximately 70 miles square, or to poults hatched from eggs originating in that area. The type has also been isolated from several captive rattlesnakes and from a capybara (large rodent) in a zoo located in the same locality. No rattlesnakes found on ranches have as yet been available for study. However, the ranch mentioned as having been the egg source for four of the six 1944 outbreaks is known to be infested with rattlesnakes. It is also known that some of the infected captive rattlesnakes originated in this area. We have not isolated the organism from natural outbreaks in chickens.

SUMMARY

Antigenic and cultural studies on a coliform organism isolated from 19 outbreaks in turkeys on 17 different ranches showed it to be a paracolon designated by Edwards *et al.* (1943) as type 10. The antigenic structure is XVIII: α_4 $\alpha_2 \times 7$. The symptoms and pathology observed in these outbreaks resembled those seen in salmonellosis of turkey poults. The mortality ranged from 5 to 70 per cent and was most severe during the first 14 days. It has been found in adult turkey carriers on 4 ranches. Agglutination tests conducted on the survivors of acute outbreaks indicate that the infection is more easily aborted than is pullorum disease. These studies add additional evidence that this group of paracolons is of pathogenic significance.

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THE ISOLATION OF *SARCINA UREAE* (BEIJERINCK) LOHNIS FROM SEA WATER

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The writer has for some years been interested in marine bacteria, particularly in connection with their role in fish spoilage and in the fouling of ships. The interesting result of the work has been to show that the bacterial flora of surface water some 5 miles offshore in the Cronulla region has a strong resemblance to a soil flora. It is the purpose of this note to record the finding of *Sarcina ureae* in surface water approximately 5 miles east of Jibbon Cape.

Sarcina ureae has been adequately described by Gibson and the identification of this organism by Beijerinck (1901) as a sporeforming *Sarcina* confirmed. Gibson (1935) has pointed out that the spores are true endospores, a fact which the present writer can fully confirm but which has not yet been admitted in *Bergey's Manual of Determinative Bacteriology*.

The strain isolated from sea water was a gram-positive *Sarcina* forming tetrads, and more rarely packets, in which a spherical endospore frequently appeared within each cell. The sporangium later disappeared, leaving the four spores united to form a tetrad, in old cultures only tetrads and diploids of spores and single spores could be seen.

A suspension of a 24-hour culture, showing no spores by microscopic examination of a stained smear, grew on shark agar after heating to 80 C° for 10 minutes and 90 C° for 5 minutes, but failed to grow after 3 minutes at 99.5 C°. A similar suspension of a 6-week-old culture, showing numerous spores, grew on shark agar after subjection to all three heat treatments. This shows that the spores are more heat-resistant than the vegetative cells, though the latter have apparently considerable resistance. It is possible that some spores were present in the slide preparation though none were observed. Gibson mentions some degree of heat resistance in the vegetative cells.

The cultures on nutrient agar, in peptone water, and in broth showed no motility in 24 hours, whereas cultures on urea agar were actively motile. Subsequent cultures on nutrient agar made from the urea agar culture were motile, though the motility was more sluggish than in cultures on urea media.

On agar the growth was at first grayish, translucent; later yellowish and semi-opaque. The growth was rather flat and the surface granular but shiny. Agar plates showed circular colonies with a regular raised margin. Gelatin showed a white growth with a glistening surface and threadlike stab growth, but no liquefaction within three weeks. In bouillon turbidity occurred, but later a granular sediment. Indole was not produced, nor were nitrates reduced to nitrites. On shark agar, ammonia production was strong in 24 hours. No

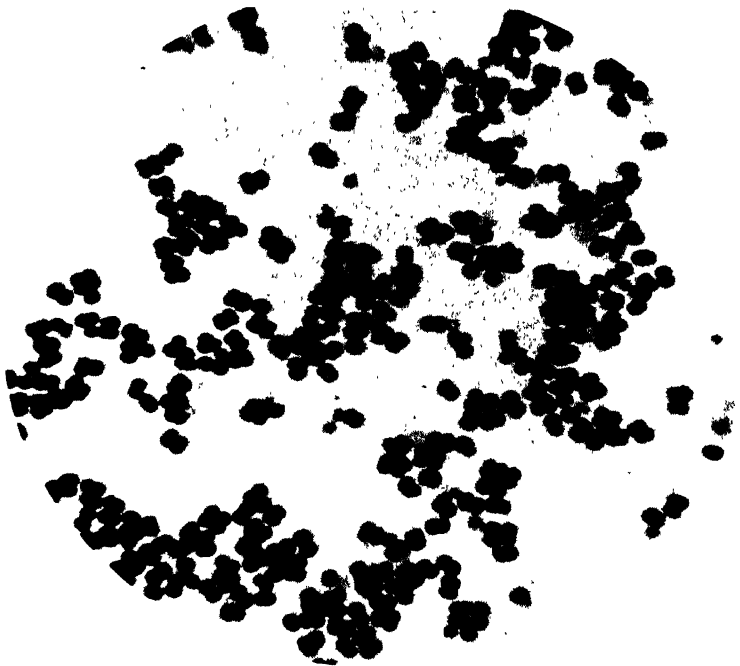


FIG. 1. PHOTOMICROGRAPH OF *Sarcina ureae*, STAINED BY GRAM'S METHOD (HUCKER'S MODIFICATION), SHOWING TETRADES

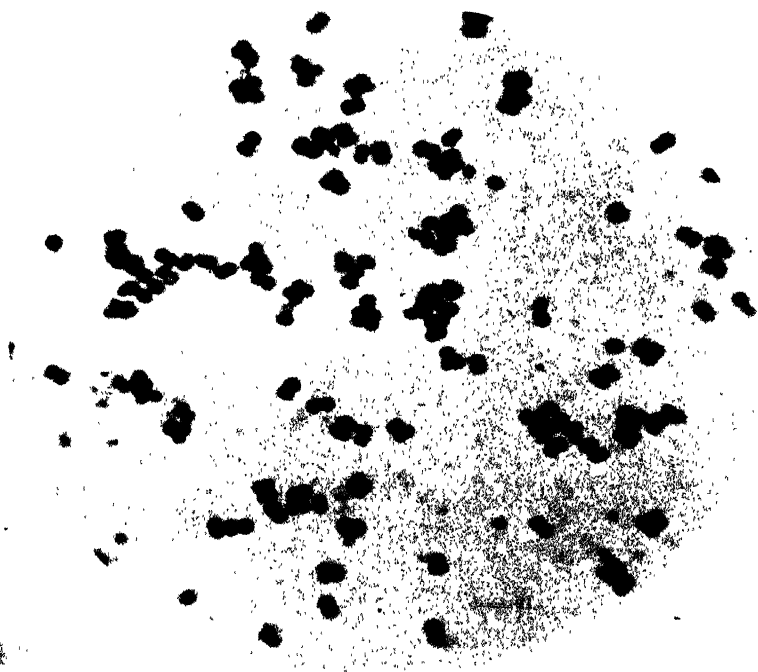


FIG. 2. PHOTOMICROGRAPH OF *Sarcina ureae*, STAINED BY MÖLLER'S METHOD, SHOWING SPORES IN PACKETS, TETRADES, AND DIPLOIDS

fermentation was observed on glucose, lactose, sucrose, maltose, mannitol, salicin, inulin, fructose, galactose, glycerol, raffinose, or xylose.

In spite of the fact that nitrates were not reduced in standard nitrate broth, there does not seem to be sufficient divergence to suggest a new species for this organism. The writer has found that nitrate reduction is likely to prove variable in other species, e.g., the *Pseudomonas* group, and should be considered in relation to the general characters of the organism. Taylor (1938) mentions this in connection with *Bacterium globiforme*.

The fact that growth on nutrient agar at pH 6.8 was readily obtained and that spore formation was rapid and frequent after 6 months of subculture is worth comment, as Gibson seems to have obtained sparse growth on nutrient agar and a falling off in spore formation on subculture.

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THE STABILITY OF PENICILLIN IN AQUEOUS SOLUTION

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In a previous publication (Benedict, Schmidt, Coghill, and Oleson, 1945), data were presented in table 1 (p. 93) which purported to be the half-lives of penicillin at various temperatures and acidities. These values were read from experimental curves and were inadvertently taken where the curves crossed the

TABLE 1

The effect of pH and temperature on the half-life of pure sodium penicillin G
(Time in hours to inactivate 50 per cent)

pH	0 C	10 C	24 C	37 C
2.0	4.25	1.30	0.31	
3.0	24	7.6	1.7	
4.0	197	52	12	
5.0	2,000*	341	92	
5.5				62
5.8			315	99
6.0			336	103
6.5			281	94
7.0			218	84
7.5			178	60
8.0			125	27.6
9.0			31.2	
10.0			9.3	
11.0			1.7	

* Estimated.

50-unit levels. Inasmuch as all experiments did not begin at the 100-unit level, the values given in the table do not represent the true half-lives. The correct values are given above in table 1.

When the corrected values are applied to the stability curves in figure 10 (p. 93), the peak of the 24 C curve will be slightly less (336 hours instead of 356) and that of the 37 C curve slightly higher (103.2 hours instead of 99).

At the time of the previous publication, it was not permissible to refer to the multiplicity of penicillins. It can now be stated, however, that the data referred to above were obtained with pure sodium penicillin G. Since that time it has been possible to obtain some of the corresponding data on three crystalline sodium penicillins, F, K, and X. Figure 1 indicates the comparative stabilities

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of these pure compounds at pH 2.0 and a temperature of 24 C. It is apparent that under these conditions, penicillin K is only about half as stable as penicillin G, the others lying midway between them. The half-lives are 7, 11, 11, and

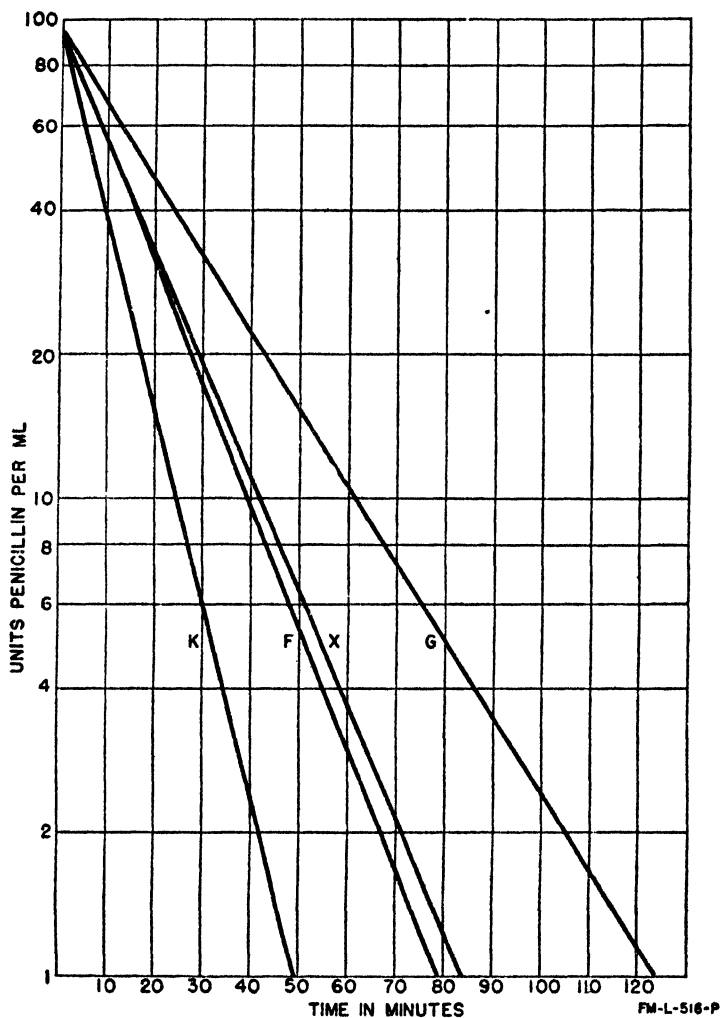


FIG. 1. STABILITY OF VARIOUS PENICILLINS AT pH 2.0 AND 24 C

18.5 minutes for penicillins K, F, X, and G, respectively. This is obviously of importance in connection with commercial recovery operations.

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THE MODE OF ACTION OF NITROFURAN COMPOUNDS. I. ACTION VERSUS STAPHYLOCOCCUS AUREUS

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Dodd and Stillman (1944) have reported that the presence of a nitro group in the 5-position of the furan ring confers antibacterial activity on a wide variety of 2-substituted furans. This earlier report demonstrated that the nitro group effect was either bactericidal or bacteriostatic, depending on the concentration present. In the present study the mode of antibacterial action of these compounds was investigated in more detail. The results, as well as comparisons between the action of various compounds and possible effect of structure on the mode of action, are presented by demonstrating graphically the effect of six typical compounds on the population of cultures of *Staphylococcus aureus*. In this instance the method proved especially valuable in demonstrating qualitative as well as previously suggested quantitative differences among the compounds. The results obtained with compounds representing the four chemical types selected by Dodd and Stillman make possible a further differentiation of nitrofuran antibacterial activity on the basis of mode of action. The six compounds used were 2-(5-nitro)-furaldehyde semicarbazone, 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, propyl-2-(5-nitro)-furoate, ethyl- β -2-(5-nitro)-furaacrylate, and 2-(5-nitro)-furfuryl propionate.

EXPERIMENTAL

The organism chosen for test purposes was *Staphylococcus aureus* Smith, obtained through the courtesy of Dr. H. J. Robinson. This organism was a rapidly growing, coagulase-positive strain. The coagulase-positive characteristic is important, since Spink (1942) has shown that coagulase-negative strains are susceptible to the antibacterial action of normal blood. We have confirmed this observation with a variety of strains of *Staphylococcus aureus*. Since it was our intention to study growth in blood, it was necessary that the test organism be resistant to the normal antibacterial activity of the blood in order to obtain a constant control and to separate the antibacterial effects induced by added nitrofurans from those encountered in the blood samples. A culture was grown by inoculating a loopful of organisms from a blood agar slant of the latest mouse passage culture into 10 ml of brain heart infusion broth containing 10 per cent of defibrinated rabbit's blood; this liquid culture was then incubated for 6 hours at 37.5 C following which it was kept at 4 C overnight. On the day of use the culture thus stored was diluted with physiological saline so as to obtain a concentration of 200,000 to 400,000 organisms per ml. The test material was inoculated with 0.1 ml of this suspension.

The antagonistic action of the nitrofurans upon the growth of this organism was studied in beef infusion broth and citrated rabbit blood. Two members of

the series were subsequently examined in rabbit serum as a test medium. The compounds were dissolved in the sterile test medium in such concentrations as were estimated from the values reported by Dodd and Stillman (1944) to give maximum, intermediate, and minimum antibacterial action within 24 hours. Five-ml portions of such solutions were pipetted into sterile pyrex tubes (100 by 12 mm) and inoculated. After obtaining a sample of 0.1 ml for the initial count of viable organisms, we placed the tubes in the incubator at 37.5 C; tests carried out in whole blood were rotated mechanically at a speed of 6 rpm throughout the test period to insure thorough mixing of cells. A sample of 0.1 ml was withdrawn at 2-hour intervals for a count of viable organisms. Counts of 48-hour samples were usually made as a check on the sterility of tubes containing the maximum concentration of the drug, but for all other concentrations observations made during the first 24 hours only are considered significant. All plates were poured with nutrient agar and incubated 24 hours before the colonies were counted.

RESULTS

Experimental data for the compounds investigated appear in the following tables. Two concentrations for each of the compounds are considered of special significance: first, the minimal concentration to give the maximal antibacterial effect, namely, a logarithmic killing effect resulting ultimately in sterilization; second, the minimal concentration to give any demonstrable effect on the growth of the organism.

The various effects of different nitrofurans upon the growth curve of the test organism, from the data in the tables, may be conveniently diagrammed as in figure 1, showing the types of curves obtained.

The curve of type V is a killing curve, showing that, under the proper conditions, these compounds may effect a sterilization of the medium. The other curves shown in figure 1 represent variations of the curve of the change in population in control cultures, which is shown as type I. We have purposely limited our consideration to the first two phases of bacterial growth, which consist of a short lag period followed by reproduction at a rate such that the population increases logarithmically. Three distinct types of growth curves different from the control, that is, type I, are shown in figure 1: type II, with growth at a reduced rate; type III, with an initial effect of decrease in population, followed by a reduced rate of growth; type IV, showing an abnormally long lag phase, followed by reproduction at a control rate. Table 7 lists the minimal concentration in a given medium at which the typical effect occurs.

The results obtained using broth as a test medium show that four compounds, namely, 2-(5-nitro)-furaldehyde semicarbazone, 2-(5-nitro)-furyl methyl ketone, 2-(5-nitro)-furoic acid, and 2-(5-nitro)-furfuryl propionate exhibit the property of sterilizing the medium. These data form the simplest case, and the results appear to be a function of concentration only. Propyl-2-(5-nitro)-furoate and ethyl- β -2-(5-nitro)-furacrylate failed to exhibit the sterilizing effect at the limit of solubility. The killing curves of type V (table 7 and figure 1) are of the same

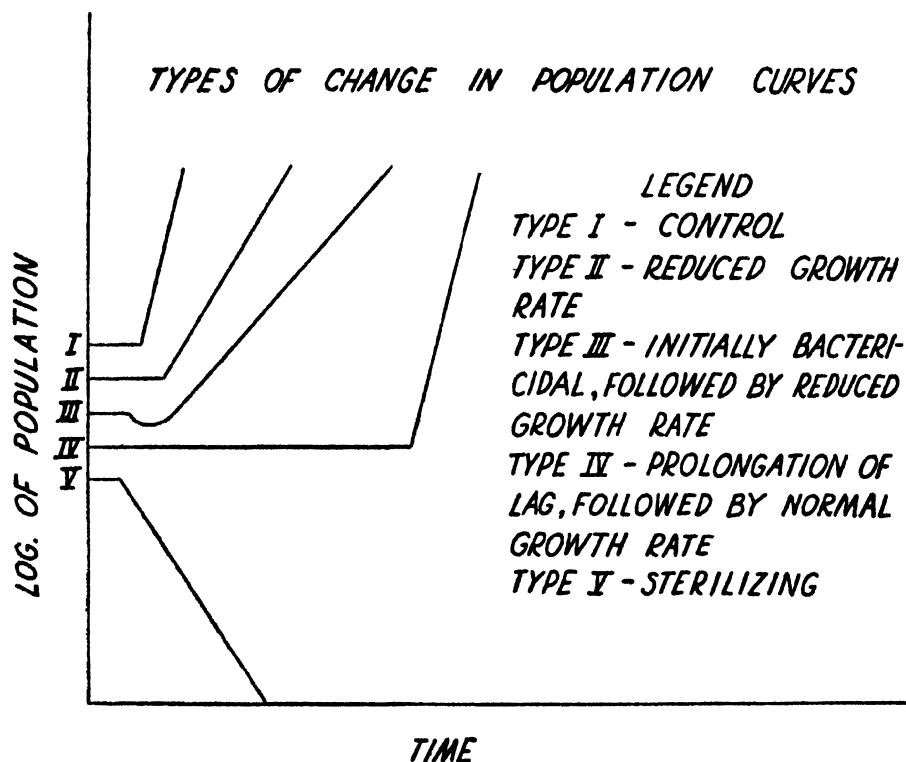
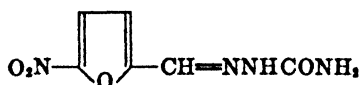


FIG. 1

TABLE 1

2-(5-Nitro)-furaldehyde semicarbazone

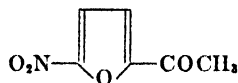


MEDIUM	DRUG CONCENTRATION	PLATE COUNT									
		Logarithm of the number of organisms per ml at indicated hour									
		0	2	4	6	8	10	12	14	16	24
Broth.....	1:200,000	4.53	4.69	4.75	4.46	4.08	<3.00	<3.00			<1.00
	1:250,000	4.79		5.00	4.70	5.00	4.90	4.75	4.64		4.90
	1:300,000	4.75		5.72	6.45	7.50	8.04	8.51	8.69		
	Control	4.78	5.38	6.88	8.38	9.18		9.55			9.55
Serum....	1:100,000	4.63	4.62	4.48	4.55	4.60	4.52	4.50			<1.00
	1:150,000	4.52	4.58	4.56	4.58	4.75	4.70	4.30			8.69
	1:200,000	4.48	4.56	4.60	4.56	5.98	6.54	6.78			8.11
	Control	4.52	4.63	6.18	6.60	7.34	8.15	8.68			8.65
Blood.....	1:50,000	4.41	4.38	4.40	4.18	3.78	3.74	3.23			<1.00
	1:75,000	4.49	4.48	4.40		4.23		4.30	4.74	5.15	8.44
	1:100,000	4.48	4.30	4.41	4.40	4.60		6.54			8.60
	Control	4.18	4.15	4.18	5.65	7.08					8.44

general type regardless of difference in the chemical structure of the compounds. Hence, we may consider the bactericidal properties conferred on the furan ring by the 5-nitro group as a common function where the effect of the 2-substitution has not reduced the solubility in the medium to a limiting value.

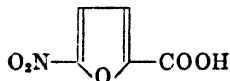
Table 7 also shows that a more complex case, in which both concentration and medium are involved, must be considered. The sterilizing properties of 2-(5-nitro)-furaldehyde semicarbazone and 2-(5-nitro)-furfuryl propionate are

TABLE 2
2-(5-Nitro)-furyl methyl ketone



MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth	1:50,000	4.70	4.61	4.34	4.15	3.86	2.30	8.73
	1:300,000	4.63	4.60	4.34	4.78	5.00	7.30	
	Control	4.66	5.15	6.62	7.62	8.57	9.64	
Blood	1:50,000	4.60	4.63	4.53	5.25	5.50	7.76	
	Control	4.54	4.52	5.30	6.40	7.00	7.91	

TABLE 3
2-(5-Nitro)-furoic acid



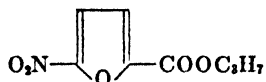
MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth	1:5,000	4.61	4.61	4.53	4.57	4.76	2.78	8.00
	1:10,000	4.60	4.82	5.08	5.48	5.30	6.53	
	Control	4.69	4.77	4.91	5.81	6.15	9.20	
Blood	1:5,000	4.62	4.62	4.87	5.76	6.48	6.48	
	Control	4.66	5.52	5.52	6.15	7.45	8.75	

maintained in both serum and blood. With these compounds the medium used for testing has modified the concentration necessary for bactericidal activity. In the case of semicarbazone, the activity is depressed by serum, more noticeably depressed by whole blood. The order of activity for 2-(5-nitro)-furfuryl propionate is the reverse of the semicarbazone. The use of blood as a medium for testing 2-(5-nitro)-furyl methyl ketone and 2-(5-nitro)-furoic acid abolished the bactericidal properties found in broth. This suggests that if nitrofurans were

examined for bactericidal properties in a simple, chemically defined medium, it might be found that the function is common to all members when sufficient solubility is maintained.

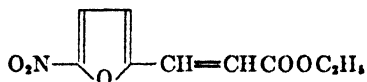
The simplest bacteriostatic activity is exhibited by 2-(5-nitro)-furoic acid, which shows only type II activity (table 7), that is, a reduction in the rate of growth. This effect depends upon both concentration and medium, since it was observed that the activity was considerably less in blood than in broth, and this

TABLE 4
Propyl-2-(5-nitro)-furoate



MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth	1:40,000	4.60	4.62	4.64	4.74	4.77	3.70	6.45
	1:100,000	4.56	4.90	5.15	5.57	5.78	8.30	
	Control	4.62	5.04	6.72		8.81	8.30	
Blood	1:10,000	4.79	4.78	6.04	6.84	7.23	8.54	8.00
	Control	4.82	4.82	6.20	7.11	7.94	8.87	

TABLE 5
Ethyl-β-2-(5-nitro)-furacrylate

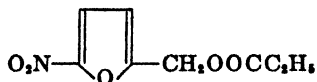


MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth . . .	1:30,000	4.74	4.62	4.57	4.58	4.69	4.15	9.08
	1:100,000	4.59	4.74	5.15	5.71	6.14	9.26	
	Control	4.62	5.04	6.72	8.00	9.35	9.53	
Blood . . .	1:20,000	4.83	4.91	5.55	6.15	6.75	7.34	8.66
	Control	4.76	4.89	5.60	6.63	7.70	8.68	

growth reduction is the only type of bacteriostasis shown by the acid. In low concentrations 2-(5-nitro)-furyl methyl ketone, propyl-2-(5-nitro)-furoate, and ethyl-β-2-(5-nitro)-furacrylate also exhibit this simple bacteriostatic action of reducing growth rate. In addition to concentration, the medium used is a factor.

A combination of an initial killing effect followed by a decreased growth rate, resulting in an ultimate bacteriostatic effect, shown in figure 1 as type III, is achieved in broth with 2-(5-nitro)-furyl methyl ketone, propyl-2-(5-nitro)-furoate, ethyl-β-2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate.

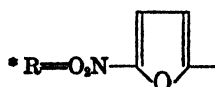
TABLE 6
2-(5-Nitro)-furfuryl propionate



MEDIUM	DRUG CONCENTRATION	PLATE COUNT						
		Logarithm of the number of organisms per ml at indicated hour						
		0	2	4	6	8	24	48
Broth.....	1:5,000	4.60	3.34	3.00	2.68	2.30	<1.00	<1.00
	1:10,000	4.58	3.92	3.99	3.92	3.91	4.69	8.93
	1:20,000	4.61	4.57	4.48	4.90	5.08	7.30	8.90
	Control	4.66	5.15	6.62	7.62	8.57	9.64	8.73
Serum.....	1:10,000	3.78	3.00	3.30	3.00	2.78	1.60	<1.00
	1:40,000	4.37	4.46	3.08	2.80	2.20	5.78	8.50
	1:80,000	4.35	4.04	5.49	6.78	7.65	8.15	8.00
	Control	4.51	4.36	6.04	7.43	8.42	9.28	9.28
Blood.....	1:40,000	4.38	3.63	2.60	2.20	1.48	<1.00	
	1:80,000	4.36	4.38	4.43	5.08	5.68	8.30	
	1:100,000	4.36	4.25	5.18	5.78	6.78	8.18	
	Control	4.38	4.25	5.23	5.90	6.80	8.38	

TABLE 7
The effect of varying concentration and medium upon the population curves

COMPOUNDS*	TYPE OF EFFECT			
	II	III	IV	V
R-CH=NNH- CONH ₂			Broth 1:300,000 Serum 1:200,000 Blood 1:100,000	Broth 1:200,000 Serum 1:100,000 Blood 1:50,000
R-COCH ₃	Blood 1:50,000	Broth 1:300,000		Broth 1:50,000
R-COOH.....	Broth 1:10,000 Blood 1:5,000			Broth 1:5,000
R-COOC ₂ H ₅	Broth 1:100,000 Blood 1:10,000	Broth 1:40,000		
RCH=CHCO- OC ₂ H ₅	Broth 1:100,000 Blood 1:20,000	Broth 1:30,000		
RCH ₂ OOCCH ₃ ..		Broth 1:20,000 Serum 1:80,000 Blood 1:100,000		Broth 1:5,000 Serum 1:10,000 Blood 1:40,000



This may be a concentration effect and represent a transition from type II to type V activity for the ketone and the propionate, since at higher concentrations

these compounds exhibit a sterilizing effect. On the other hand, if both reproduction and killing were being effected simultaneously, with only a small proportion of each generation being killed, then the population curve would show an initial drop, followed by an increase resembling growth at a reduced rate. Similar factors in the mode of action of penicillin were discussed by Lee, Foley, and Epstein (1944). This type III effect is also influenced by the medium. None of the compounds exhibited the phenomenon in blood, with the exception

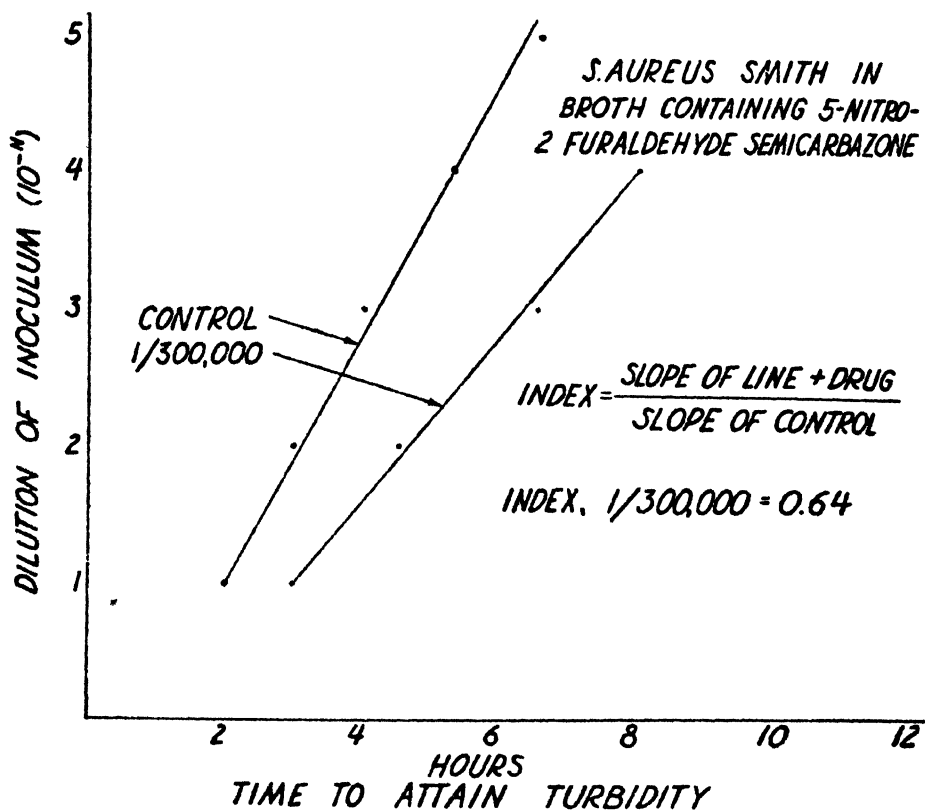


FIG. 2

of 2-(5-nitro)-furfuryl propionate. With this compound the activity is greater in serum and in blood, in the order named.

The compound 2-(5-nitro)-furaldehyde semicarbazone exerts an entirely different mechanism of bacteriostatic action. With bacteriostatic concentrations of this drug, the growth curve (figure 1, type IV) shows an abnormal prolongation of the lag phase followed by reproduction at the control rate. We have tested this effect in three separate media: broth, serum, and blood. The same mechanism holds, regardless of the medium. However, the effectiveness of this semicarbazone is reduced by both serum and blood, the latter exhibiting the greater antagonistic effect.

We have also determined the type of growth curve obtained with bacteriostatic concentrations of this compound greater than the minimal bacteriostatic concentration but less than the amount necessary to kill, in any given medium. These increased concentrations do not affect the mode of action other than to prolong the time that the organism will remain in the lag phase.

A similar phenomenon in the bacteriostatic action of crystal violet has previously been shown by Dubos (1929), Ingraham (1933), and Hoffman and Rahn (1944). We have applied the test described by Ingraham (1933). A series of tubes each containing the same given concentration of the compound in nutrient broth is inoculated with a tenfold dilution series of the test organism. If the rate of growth is undisturbed, but the lag phase prolonged, the time for each successive tube to reach just visible turbidity when plotted against the dilution number of that tube, and hence the dilution of the inoculum, should give a straight line. In this manner the length of the lag phase is made the controlling factor. The results from a representative experiment using 2-(5-nitro)-furaldehyde semicarbazone at a concentration of 1:300,000 in broth, inoculated with *S. aureus*, appear as figure 2; the straight-line relationship is obvious.

DISCUSSION

Within a group of six widely different chemical compounds of the nitrofurans class, it has been shown that the 5-nitro group may confer killing properties to four of the compounds. In broth the chemical nature of the 2-position substitution in the compounds seemed to bear only a quantitative relationship to the activity. The property is therefore a function of the concentration and is apparently absent in the other two compounds because the concentration necessary to reach the sterilizing effect exceeds the maximum solubility in the medium. Moreover, the test medium employed may affect the potency of this killing action. The ability of blood to produce these changes is greater than that of serum. The effect is depressant with 2-(5-nitro)-furaldehyde semicarbazone and completely abolishes the sterilizing activity with 2-(5-nitro)-furfuryl methyl ketone and 2-(5-nitro)-furoic acid. The bactericidal property of 2-(5-nitro)-furfuryl propionate is exalted both in serum and in blood.

The bacteriostatic activity of these compounds may be divided on the basis of the effect on bacterial growth curves. One group, consisting of 2-(5-nitro)-furfuryl methyl ketone, 2-(5-nitro)-furoic acid, propyl-2-(5-nitro)-furoate, ethyl- β -2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate, acts as a growth rate depressant. In this group the nature of the side chain in the 2-position on the furan ring exerts only a quantitative effect. With a given medium, the compounds exhibiting this type of activity differ only in the concentration necessary to produce a bacteriostatic effect. The activity of all except one may be reduced by employing serum or whole blood as a test medium, the latter exerting a more antagonistic effect. Dodd and Stillman (1944) have shown that in a simple, chemically defined medium the antibacterial activity of the nitrofurans against *Escherichia coli* is even greater than the activity in broth. These facts suggest that nitrofurans affect adversely a metabolic function of the organism; the nature

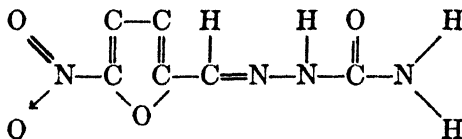
of this effect, or the substrate involved, is as yet unknown. The exception to the depressant action of blood and serum is 2-(5-nitro)-furfuryl propionate. The activity of this compound appears to be enhanced by both serum and blood. No explanation can be given for this unusual effect, unless we assume conversion to a more active compound. Obviously, this explanation involves an effect of the 2-position substituent of the furan ring on the activity and would remove this compound from the group as defined.

A variation of this depressed growth rate type of bacteriostatic activity was exhibited in broth by 2-(5-nitro)-furyl ketone, propyl-2-(5-nitro)-furoate, ethyl- β -2-(5-nitro)-furacrylate, and 2-(5-nitro)-furfuryl propionate. Upon examination of the population at intervals after the inoculation of the drug-containing medium, a slight decrease, followed by an increase at a reduced growth rate, is noted. Obviously, the concentration necessary to demonstrate this is an important factor. It may be that this represents a transition point from bactericidal to simple bacteriostatic activity. If so, then structure is not involved. However, a partial or proportional killing process would yield the same type of curve if only a small percentage of each succeeding generation were killed. Since all of the compounds tested do not demonstrate this type of activity, then the nature of furan ring substitutions other than the nitro group modifies the effect, if we assume the second explanation. The medium used in testing also contributes an effect; only the propionate gives this type of curve in serum and blood.

A distinctly different mode of bacteriostatic action was demonstrated with 2-(5-nitro)-furaldehyde semicarbazone. This compound apparently has no effect on the rate of growth, once growth is initiated; it does, however, prolong the time of the lag phase, in which the population remains stationary. This phase of growth, distinguished by the lack of reproduction, is generally conceded to be a period of intense vital activity of the organism. Whether the semicarbazone affects some vital process other than reproduction in such a fashion as to prolong the time to attain maturity, or merely interferes with cell division, cannot at this time be stated definitely. However, erratic results observed with coagulase-negative staphylococci suggest that metabolic processes during the lag phase are affected. Details of this activity are being studied and will be published later. We have found that the only effect of increasing the bacteriostatic concentration of the semicarbazone in a given medium is to increase the length of the lag period. The basic features of the mode of action remain the same. This favors the explanation that the increase in lag period is merely a poisoning of the organism. The point is an important one, for it is theoretically possible that in this case we have a drug which for at least a period maintains a rate of kill equal to the rate of growth. If this be true, however, then it would be expected that even slight increases in concentration would depress the population as an increasing linear function of the concentration. This does not agree with the observed facts.

Dubos (1929), in postulating a mechanism for a similar lag prolongation with crystal violet, ascribed the effect to the ability of the dye to poison the oxidation-reduction potential at an unfavorable point; the idea has been elaborated by

Such unique properties must be due, at least in part, to the qualitative contribution of the structure of the semicarbazone chain in the 2-position of the furan ring to the antibacterial properties of the 5-nitro furan radical. Possibly the semicarbazone chain has conferred unusual electrobiological properties to the nitrofuran ring. A poisoning of potential would result if the nitrofuran semicarbazone were preferentially reduced at any point in the hydrogen transport system of bacterial respiration. Moreover, this interference with a normal metabolic system could be manifest as an antibacterial property of the compound. The structure of this semicarbazone



As a chemical compound, this semicarbazone in aqueous solution can be reduced by a variety of methods, including the action of sodium hydrosulfite. Preliminary results indicate the nitro group as the first point of attack. The extent of reduction and the characteristics of reduction products are at present being studied.

One compound, 2-nitro-(5-nitro)-furaldehyde semicarbazone, was unique in its mode of bacteriostatic action. Activity was shown in the lag phase of growth.

The rate of reproduction, once initiated, was unaffected by the compound. Both concentration and medium affect the activity. The possible contribution of structure to this mode of action was discussed.

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SOIL ENRICHMENT AND DEVELOPMENT OF ANTAGONISTIC MICROORGANISMS^{1,2}

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INTRODUCTORY AND HISTORICAL

The soil enrichment method is one of the most common procedures employed by the microbiologist to isolate specific microorganisms concerned in certain processes of importance to soil fertility or of special interest for other reasons. In a broad sense, the enrichment method involves three distinct steps, which may be briefly summarized as follows:

(1) Fresh soil is placed in the laboratory under favorable conditions of temperature, moisture, and aeration. It is then treated or "enriched" with a simple or complex organic or inorganic substance, in order to stimulate the development of specific organisms capable of utilizing this substance. Thus, if one is interested in isolating nitrifying, cellulose-decomposing, or petroleum-oxidizing bacteria, the soil is treated with an ammonium salt, with a cellulosic material, or with petroleum. Sufficient water is added to provide favorable moisture, about 50 to 70 per cent of the moisture-holding capacity, and the soil is incubated at a suitable temperature. These treatments result in the selective development in the soil of a specific microbiological population capable of bringing about the process in question. Frequently certain secondary reactions of an undesirable kind occur as a result of a given treatment. These may affect unfavorably the development of the desired organisms. This is true, for example, of the accumulation of acid in soil treated with an ammonium salt; such a condition can be counteracted by the addition of CaCO_3 .

(2) The second step consists in the isolation of the specific organisms. This can be done by the use of ordinary bacteriological media or special selective media. For the latter, a medium containing the particular material which was used for the soil enrichment may be employed. This medium is inoculated with the enriched soil, although unenriched or fresh soil is also commonly used for this purpose. After proper incubation, the medium, whether solid or liquid, will show the development of organisms with the capacity of bringing about the desired reaction. Repeated transfers of such enriched cultures may have to be made to fresh lots of the selective medium before an active culture of the organism which produces the desired reaction is obtained.

(3) The final isolation of the organism in pure culture is now undertaken. For this purpose, either common or selective media containing the given sub-

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strate as a major nutrient are employed, depending on the nature of the particular organism involved in the given reaction and its ability to grow on different media. The nature of the organism will influence greatly the final steps undertaken for the purpose of isolating the pure culture.

These methods were first used by some of the early masters in the field of microbiology, notably Winogradsky and Beijerinck. Many of the important soil and water bacteria have thus been isolated. The extensive use of such techniques by Beijerinck led to the preparation, in the form of a treatise (Stockhausen, 1907), of a summary of the principles underlying these methods.

The application of the enrichment methods to the isolation from soil of organisms capable of destroying disease-producing bacteria was first made by Dubos and Avery (1931). In order to obtain organisms capable of decomposing the capsular carbohydrate of pneumococci, they used a medium containing the particular carbohydrate or its derivative as the major source of energy. More recently Dubos (1939) applied the soil enrichment method for the purpose of isolating organisms capable of destroying various disease-producing cocci. A mixed soil was treated at irregular intervals with living washed cells of staphylococci, pneumococci, and streptococci for a period of 2 years. This enriched soil was then inoculated into a special medium consisting of suspensions of living bacteria in mineral salt solutions. Several transfers were thus made into fresh lots of the same medium, a procedure which led to the selective development of the desired organism capable of killing the bacteria in suspension. Final isolation of the antagonist was accomplished on ordinary peptone agar. This resulted in the isolation of *Bacillus brevis*, which has the capacity of producing two antibiotic substances, namely, gramicidin and tyrocidine.

The enrichment method was also utilized by Waksman and Woodruff (1940) in the isolation of organisms with the capacity of inhibiting the growth of other organisms or of actually killing them. These authors made extensive use of the third step in the enrichment procedure which consists of adding living bacterial cells to an agar medium. The theory underlying this method is that only those organisms capable of attacking the bacteria added to the medium would develop.

Although the occasional usefulness of the enrichment method for the purpose of isolating antagonistic organisms cannot be questioned, it still remains to be determined to what extent the classical enrichment method can be logically applied to the isolation of microbial antagonists yielding promising antibiotic substances. No such doubt exists in regard to the value of the method in the isolation of organisms capable of bringing about simple reactions, such as oxidation of an ammonium salt, decomposition of cellulose, oxidation of a hydrocarbon, or even hydrolysis of bacterial polysaccharides. There is some doubt, however, about the justification of the enrichment principle in the isolation of organisms which produce substances possessing bacteriostatic and bactericidal properties. The reason for this is the fact that the antibiotic substances affect bacteria, not by a mechanism of digestion or hydrolysis, but by means of reactions that vary for each agent. One need only mention interference with the enzyme mechanism of the bacterial cell, the substitution of a poisonous principle

for a metabolite required by the cell, or the effect of the antibiotic upon the reproductive capacity of the cell, upon its surface tension, or upon some other mechanism essential to the life of the cell. Such reactions are not necessarily favored by the use of bacteria as a substrate for the development of microorganisms capable of producing specific inhibitive agents.

Most of the organisms which produce antibiotic substances, including both the penicillin-producing *Penicillium notatum* and the streptomycin-forming *Streptomyces griseus*, have been isolated neither by the direct enrichment of soil nor by the use of bacteria-enriched media. Even in the case of tyrothricin-producing bacteria, the need for special enrichment methods has been questioned. Stokes and Woodward (1942) demonstrated, for example, that such bacteria can easily be isolated from ordinary fresh field or garden soils by the mere planting of low dilutions of such soils on ordinary nutrient agar; the colonies of those bacteria that possess antibacterial properties will produce a clear zone in which the growth of other bacteria is inhibited; such organisms isolated from the plate are usually found to be capable of producing tyrothricin.

As long as the usefulness of the soil enrichment procedure for isolating organisms possessing antibacterial properties remains in doubt, there is room for speculation concerning the application of this method to the isolation of antagonistic organisms. Just how or why should the introduction into soil of living bacterial cells favor the development of organisms capable of producing substances which have a selective inhibitive effect upon those particular bacteria by interfering with their nutrition or affecting their enzyme mechanisms? Since such living bacteria are not actually used by these organisms for nutrition and since they are not, therefore, actual substrates for the development of antagonists, one is justified in questioning whether the ability of certain microorganisms to produce antibiotic substances is a result of a reaction indispensable to the survival of the organism in the soil or is merely an accidental characteristic, similar to many other properties possessed by living systems for which no explanation of purposefulness has as yet been found.

Rhines (1935) demonstrated that the introduction of *Mycobacterium avium* into the soil resulted in the gradual destruction of the organism; this was apparently associated with the presence in the soil of certain specific microorganisms. Waksman and Foster (1937) emphasized the fact that the ability of an organism to become established in the soil, even when sterile soil is used as the medium and the pure culture added, depends upon the presence of other organisms, which, once established, will prevent the survival of the newly introduced form. Attention should also be directed to the fact that not all organisms added to the soil die out rapidly in that medium. The ability of a given organism to survive when added to fresh soil is influenced by the reaction of the soil and the abundance and nature of the organic matter, as was shown by Katznelson (1940). Out of a large number of bacteria, fungi, and actinomycetes added to the soil, only *Pseudomonas fluorescens*, *Fusarium culmorum*, and *Actinomyces cellulosa* died out completely, whereas all the others survived, although in reduced numbers.

In order to throw further light upon this problem, the following experiments were undertaken.

EXPERIMENTAL

Soil used. A rather poor field soil, of pH 6.5, was sieved to remove stones and coarser plant residues, and placed in small earthenware pots. The moisture of the soil was adjusted to optimum, namely, 60 per cent of the moisture-holding capacity. Throughout the experiment the moisture content of the soil was found to vary from 11.5 to 14.2 per cent, a percentage which was considered favorable for the development of aerobic organisms. The pots were divided into three groups and treated as follows: (1) No bacteria added (control soil). (2) Suspension of washed living cells of *Sarcina lutea* added. (3) Suspensions of washed *Escherichia coli* cells added. The bacteria were grown on agar surfaces and 2- to 4-day-old cultures were used.

The pots were covered with glass plates and incubated at 28 C. Fresh bacterial suspensions were added repeatedly to the same pots, about every 7 to 10 days. After each addition, the pots were left uncovered for 1 to 3 days to allow the evaporation of the excess moisture that had been added with the bacterial suspension.

Microbial counts. The soils were plated out at various intervals. Two different media were used, namely, egg albumin agar for the counts of bacteria and actinomycetes, and glucose peptone agar containing 0.3 per cent H_2BO_3 , for the fungi. The latter was a modification of Tyner's (1944) medium. Counts were made before each addition of the fresh suspension of bacteria to the soil; the preparation of the soil dilutions, the incubation of the plates, and the colony counts were conducted in accordance with usual bacteriological techniques.

In some cases, special supplementary media were also employed. For the soil enriched with *S. lutea*, the number surviving in the soil could be determined by the use of ordinary nutrient agar; the yellow characteristic colonies of this organism were readily distinguished. In order to determine the abundance of various antagonists, two washed agar media were used, one containing *S. lutea* cells, and the other *E. coli* cells. On the *S. lutea* plates, those colonies producing clear zones were considered as antagonists and counted as such. In the case of *E. coli* plates, however, clear zones are rarely formed, and, consequently, all the colonies developing on the plates were counted. To what extent these may be considered as antagonists remains to be determined.

The periodic counts of the different groups of organisms showed considerable fluctuation. This was due to a number of factors. When a soil is brought from the field into the laboratory, a change in numbers of microorganisms results, as was brought out in detail in earlier studies (Waksman and Starkey, 1923). It is also known that the addition to soil of fresh organic matter results in a marked increase in microbial numbers, and frequently in a change in the proportional distribution of different organisms (Waksman and Starkey, 1924). One would expect, therefore, that the addition of fresh bacterial cells to the soil should also bring about changes, not only in total numbers, but also in the relative

distribution of the different groups of organisms. At the different periods, certain organisms appeared and later disappeared. At 15 days, for example, there appeared on the egg albumin agar plates, from the *S. lutea*-treated soils, large numbers of a bacterium not noticeably present in the other two soils. This organism, a gram-negative rod, produced raised, glistening, semiopaque or translucent, watery, and somewhat mucoid colonies, varying in diameter from about 1 to 12 mm. This particular organism made up, at the end of the 15-day incubation of the soil, 20 to 25 per cent of the colonies developing on the plates. It was also present in very large numbers after 23 and 31 days. After 40 days, however, it was found only in much reduced numbers, and after 66 days it completely disappeared from that soil. This organism was tested on a variety of media for antibiotic activity, the agar streak method being used, but was found to be completely inactive against the gram-positive and gram-negative test bacteria used.

TABLE 1
Numbers of microorganisms in control soil

AGE	ORGANISMS PER G OF DRY SOIL		
	Bacteria	Actinomycetes	Fungi
<i>days</i>	<i>thousands</i>	<i>thousands</i>	<i>thousands</i>
1	3,220	2,000	28
2	2,550	1,160	
8	1,490	1,740	28
15	7,360	1,840	20
23	5,730	1,720	17
31	3,650	1,140	18
40	8,020	2,600	32

Numbers of microorganisms. The numbers of microorganisms, as determined in the control soil (table 1), show at first a drop, followed by a rise. The changes were not gradual but underwent marked fluctuations, more for the bacteria than for the actinomycetes, and least for the fungi. The total numbers were rather low, undoubtedly because this was a rather poor soil.

The soil that received several additions of *S. lutea* cells showed a marked increase in the numbers of bacteria. This increase was not due to the establishment of the *S. lutea* in the soil, since it rapidly died out (table 2). There was an increase in the numbers of this organism only immediately after its first introduction into the soil, when larger numbers were found than were added to the soil. This may be due to the breakup of the bacterial clumps in the soil rather than to their multiplication. Further repeated additions of *S. lutea* to the soil led to the rapid disappearance of this organism. Whether this is due to the establishment in the soil thus treated of organisms which are responsible for the disappearance of the added bacteria still remains to be determined. When the number of antagonists in this soil was determined by the *S. lutea* agar plate (table 4), only a slight increase was obtained, as compared with the control soil.

A similar increase was obtained in the number of antagonists against *S. lutea* in the soil enriched with *E. coli*. These results are comparable to those reported previously (Waksman and Woodruff, 1940a, 1940b) on the enrichment of soil with *E. coli*.

The effect of the addition of *S. lutea* cells upon the development of actinomycetes and fungi was rather limited. The greatest effect was on the total numbers

TABLE 2
Microbiological population of soil enriched with Sarcina lutea

AGE	VIABLE CELLS ADDED PER 1 G OF SOIL	ORGANISMS PER G OF DRY SOIL			
		<i>S. lutea</i> -like colonies	Bacteria	Actinomycetes	Fungi
days	thousands	thousands	thousands	thousands	thousands
0	50,000		3,220	2,000	28
1		163,000	7,880	2,920	22
2		16,200	134,000	2,680	
8	12,000	<1,000	37,100	1,500	32
15	56,000	<100	34,500	1,470	24
23	320,000	<100	102,000	2,770	23
31	681,000	<100	80,900	2,550	41
40		<100	6,980	1,500	25

TABLE 3
Microbiological population of soil enriched with Escherichia coli

AGE	VIABLE <i>E. coli</i> CELLS ADDED PER 1 G OF SOIL	ORGANISMS PER G OF DRY SOIL			
		Colonlike organisms	Bacteria	Actinomycetes*	Fungi
days	thousands	thousands	thousands	thousands	thousands
0	2,800,000		3,220	2,000	28
1		>1,080,000	>1,060,000		22
2					
8	2,000,000	970	4,850	1,150	26
15	4,000,000	1,400	47,500	1,840	20
23	2,800,000	201	25,600	2,230	46
31	2,000,000	2,480	53,200	2,430	51
40		6,770	104,000	1,380	49

* The count of actinomycetes could not be accurately made on egg albumin medium because the dilution plates exhibited practically all *E. coli* colonies, which covered almost the entire surface.

of bacteria, the rise in numbers being very marked after 2 and 23 days, followed by a considerable drop. It is difficult to determine, however, whether these bacteria were responsible for the destruction of the *S. lutea* cells or whether they merely utilized the dead cells as food material. It is known (Waksman and Starkey, 1924) that protein-rich materials favor multiplication of bacteria in the soil.

The enrichment of soil with large numbers of *E. coli* cells (table 3) also stim-

ulated the bacterial population and had a comparatively limited effect upon the actinomycetes and the fungi. The *E. coli* cells, like the *S. lutea*, died out rapidly, as was determined by plating on the EMB medium. Whether the bacteria multiplied at the expense of the dead *E. coli* cells or whether they were responsible for killing these cells still remains to be determined.

A study of the possible antagonistic organisms developing in the soil enriched with *E. coli* shows (table 5) a definite increase in the number of total organisms, mostly fungi, capable of developing on the *E. coli* plates. There was no proof, however, that these organisms are important antagonists and are not merely forms that have been selected or adapted to grow on the dead *E. coli* cells in the soil enriched with these cells.

When first plated out, each of the three soils displayed a normally heterogeneous mold population. The first difference was observed on the 31-day plating. The soils enriched with the bacteria contained much greater numbers

TABLE 4

Lysis-producing colonies of organisms developing on Sarcina lutea plates

Washed agar *S. lutea* plates incubated 6 days at 28 C and colonies producing clear zones counted. Results expressed in thousands per 1 g of soil

TREATMENT OF SOIL	AGE OF SOIL, DAYS OF INCUBATION*			
	8	23	32	41
	Colonies of antagonistic organisms			
Control soil	550	400	420	980
Soil enriched with <i>S. lutea</i>	1,510	600	2,100	900
Soil enriched with <i>E. coli</i>	2,300	380	480	4,100

* See table 1 for dates of enrichment of soil and number of living bacterial cells added per 1 g of soil.

of certain uniform pink *Fusaria* than the control soil. On the 40-day plating, the increased development of such *Fusaria* in the soil enriched with *S. lutea* was still evident; there was also a small but definite increase of fungi belonging to the *Penicillium luteum-purpurogenum* group. The soil enriched with *E. coli* contained even larger numbers of the pink *Fusaria* than the soil enriched with *S. lutea*; it did not, however, show any stimulation of the *P. luteum-purpurogenum* group. On the 66-day plating, the *E. coli* soil still contained larger numbers of pink *Fusaria* than the control soil, but fewer than in the previous plating. The *Fusaria* isolated from the plates had considerable antibiotic activity against gram-positive bacteria, but not against *E. coli*. The control soil contained some of these pink *Fusaria*. It may also be of interest to report here the fact that soils enriched with living or dead cells of *Mycobacterium tuberculosis* favored extensive development of certain actinomycetes, such as *Streptomyces coelicolor*. This organism made up 20 per cent of all the colonies developing on the plate

prepared from such enriched soils; however, it had no antibiotic effect upon either *M. tuberculosis* or other bacteria.

In order to determine the significance of these results in terms of well-known antagonistic organisms, three series of washed agar plates treated with suspensions of cells of (1) *E. coli*, (2) *S. lutea*, and (3) *Bacillus subtilis* were inoculated with cultures of a selected group of organisms known to be important producers of antibiotic substances. The growth of these organisms and the formation of clear zones were studied. The results (table 6) show that none of the antagonists tested on the agar plate containing washed bacterial cells produced any clear zones on the *E. coli* plates, although some, notably the fungi, made good growth on such plates. On the *S. lutea* plates, however, all of the organisms tested made good growth, and a number of them also produced markedly clear zones. The difference in the effects of the two types of cells

TABLE 5

Colonies of organisms developing on E. coli plates

Washed agar *E. coli* plates incubated for 6 days at 28 C, and total colonies counted.
Thousands per 1 g of soil

TREATMENT OF SOIL	AGE OF SOIL, DAYS OF INCUBATION*							
	8		23		41		66	
	Colonies of organisms							
	TC†	FC	TC	FC	TC	FC	TC	FC
Control soil	15	15	20	15	50	30	90	50
Soil enriched with <i>S. lutea</i>	30	30	70	50	270	190	40	40
Soil enriched with <i>E. coli</i>	40	40	100	100	200	160	120	110

* Same footnote as table 4.

† TC = total colonies on plate; FC = fungus colonies.

is due entirely to the ability of *S. lutea* to undergo rapid lysis, thus supplying larger amounts of nutrients for the growth of the antagonists and incidentally forming lytic zones. The results with *B. subtilis* were analogous to those obtained on the *S. lutea* plates. The two gram-positive bacteria allowed good growth of the antagonists and the production of clear zones.

It is of particular interest to note that of the three antagonistic bacteria tested, the two sporeformers, especially *B. brevis*, produced clear zones on the *S. lutea* and *B. subtilis* agar, but *Pseudomonas aeruginosa* had no such reaction, although it made as good growth as the sporeformers.

The five cultures of actinomycetes did not grow at all on the *E. coli* plates, in spite of the fact that two of these cultures, namely, *S. griseus* and *Streptomyces lavendulae*, are able to produce antibiotic substances (streptomycin and strepto-

thricin, respectively) that have a marked bacteriostatic and bactericidal effect on *E. coli*. On the plates containing the gram-positive bacteria, *Streptomyces antibioticus* made some growth but produced no clear zones, although this organism produces a substance (actinomycin) which is highly active against those bacteria.

The five cultures of fungi grew well on the *E. coli* plates but produced no zones. They also grew well on the plates enriched with the gram-positive

TABLE 6

*Growth of antagonistic organisms on bacterial washed agar media and lysis of bacteria**

ANTAGONISTIC MICROORGANISM	MEDIUM CONTAINING WASHED CELLS OF					
	<i>Escherichia coli</i>		<i>Sarcina lutea</i>		<i>Bacillus subtilis</i>	
	Growth†	Lysis	Growth	Lysis	Growth	Lysis
I Bacteria						
<i>B. brevis</i>	0	0	+++	+++	++++	++++
<i>B. simplex</i>	0	0	+++	+	+++	++
<i>P. aeruginosa</i>	0	0	++++	0	+++	0
II Actinomycetes						
<i>S. antibioticus</i>	0	0	++++	0	++	0
<i>S. griseus</i>	0	0	+++	+	++	++
<i>S. lavendulae</i>	0	0	++++	+	+++	++
<i>Micromonospora</i> sp.	0	0	++++	+++	0	0
<i>N. gardneri</i>	0	0			+++	++++
III Fungi						
<i>A. clavatus</i>	+++	0	++++	++++	+++	++++
<i>A. flavus</i>	+++	0	+++	0	+++	0
<i>A. fumigatus</i>	+++	0	+++	++	+++	+++
<i>Gliocladium</i> sp.	+++	0	++++	++	+++	0
<i>P. notatum</i>	+++	0	++++	±	0	0

* The basic medium consisted of 1% glucose, 0.05% K_2HPO_4 , and 1.5% washed agar in distilled H_2O . One series of plates contained a suspension of washed, viable *E. coli* cells distributed throughout the agar; another series contained *S. lutea* cells; to a third *B. subtilis* cells were added. All plates were incubated at 28°C and were examined after 5 days.

† 0 = no growth of antagonist or lysis of test bacterium as shown by formation of clear zone on plate, ± = trace, + to ++++ = increasing amounts of growth or lysis.

bacteria, except *P. notatum* on the *B. subtilis* plate. The production of the clear zones varied greatly, however. *Aspergillus clavatus*, which forms clavacin, produced very wide zones. *Aspergillus flavus* and *P. notatum*, both of which produce penicillin, formed no zones at all.

One must, therefore, conclude that the production or lack of production of zones on the bacterial agar plate is no proof at all of the ability of the organism to produce an antibiotic substance, and certainly throws no light upon the nature of the substance formed by the organism.

DISCUSSION

Various attempts have been made to interpret the production of antibiotic substances by microorganisms in terms of the Darwinian concept, namely, "the struggle for existence and survival of the fittest." This concept, after having penetrated into many phases of biology, has not failed to make its appearance also in microbiology. However, the various facts submitted in support of the application of this principle to some phases of microbiology hardly constitute sufficient evidence. Whereas this theory may be fully justified when applied to the animal kingdom and to higher plants, it does not always fit in when applied to microbial associations. The survival of a microbe in the soil may be due to several factors: (a) its ability to utilize some of the chemical constituents in the soil more readily than can other organisms already present; (b) the ability of the particular microbe to tolerate soil conditions of reaction, moisture, and temperature which may prove to be less favorable to other organisms; and (c) its capacity to produce substances inhibitive to the growth of other organisms. The last factor may be conceived as rather an incidental property of one organism that gives it a certain advantage over others. In view of the fact that the action of such substances is usually not of a generalized but rather of a selective nature, there will at best result the inhibition of growth of only certain specific organisms, but not of many others.

Although one may speak of competition for nutrients in terms of "struggle for existence" when two organisms are able to utilize the same substrate, this concept can hardly apply to the possible interaction between two fungi, such as *Penicillium notatum* and *Trichoderma koningi*, in the soil; the first of these is unable to utilize cellulose, whereas the second is one of the strongest cellulose-decomposing organisms. An even more striking example can be found in a comparison of *B. subtilis* or *E. coli* with the *Cytophaga* group of bacteria: the last is able to use cellulose, whereas the first two organisms are totally unable to attack cellulose.

In the early days of microbiology, when knowledge of antagonism among microorganisms first appeared in the work of Emmerich and Garré (Waksman, 1945), the terms "antibiosis" and "struggle for existence" were virtually synonymous. Papacostas and Gaté (1928) drew attention to the fact that such an interpretation as applied to microorganisms would be justified only if one organism were to secrete enzymes that would actually destroy the other organism. They illustrated this by the effect of the venom of a serpent, which serves to kill the prey before it is consumed, as against the production by one microbe of a substance which interferes with the life of another microbe without necessarily benefiting its producer by such an effect. The numerous interrelations among microorganisms in natural environments include: (1) favorable effects, which range from the consumption of oxygen by an aerobe, thereby making conditions favorable to an anaerobe, to the production of growth-promoting substances by one organism necessary for the growth of another; (2) strict "symbiosis"; and (3) unfavorable effects, which range from the undesirable action of an acid produced by one organism upon the growth of another and specific nutrient

competition, to the phenomenon of antagonism or "antibiosis," in which one organism produces substances exerting an unfavorable effect upon the growth of another. The many phenomena of "metabiosis," and the living together of two organisms without any apparent effect of one upon the other, fall between.

Papacostas and Gaté suggested, therefore, complete abandonment of the application of the Darwinian concept to the interactions among microorganisms. In an attempt to clarify or simplify inhibitive phenomena, however, they suggested the application of the term "antibiosis" to mixed cultures (*in vitro*) and the term "antagonism" to mixed infections (*in vivo*). Such designations would tend to suggest that the interactions among microorganisms in the test tube are distinct from those in the body. Recent evidence in the field of antibiotics does not bear this out. If there is any justification for differentiating between these two terms, the distinction suggested by Waksman (1945) might be made: the term "antagonism" might be used to apply to the complex unfavorable effects of one living system upon another, when the mechanisms involved are not as yet clearly understood, and "antibiosis" to describe the phenomena of specific selective activities of chemical substances, the antibiotic agents produced by one organism which act upon another.

SUMMARY

The results of a study of the enrichment of soil with suspensions of living bacteria brought out the fact that such soil enrichment affects only to a limited extent the microbiological population of the soil, as is determined by the plate method. Any increase in the development of specific groups of organisms in the soil may merely indicate the ability of such organisms to attack the living or dead bacterial cell substance. There is still lacking sufficient proof that any organisms thus stimulated in the soil are antagonistic to the added cells or produce antibiotic agents active against the enrichment bacteria.

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THE MANNITOL-NEGATIVE SHIGELLA GROUP

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A primary division of the human pathogens of the genus *Shigella* is based upon the fermentation of mannitol, a widely used test of considerable practical value. By this means the nonfermenting *S. dysenteriae* and *S. ambigua* species may be differentiated from the more common *S. paradysenteriae*, *S. sonnei*, and *S. alkalescens*.

In the mannitol-negative group the generally used American classification (Bergey's Manual, 1939) includes the species *S. septicemia* and *S. minutissima*. In the diagnosis of human disease, only *S. dysenteriae* and *S. ambigua* have been considered pathogenic. Organisms which differed from these two in various characteristics have been encountered, however, and recently descriptions of new non-mannitol-fermenting *Shigella* types occurring in India, North Africa, the United States, and England have been published. Sachs (1943) reported eight serological types among strains isolated in Egypt and India which he believed belonged to *Shigella*. These included four of the types (771, 902, 1030, 1167) previously described by Large and Sankaran (1934). Christensen and Gowen (1944) isolated two serological types from army personnel in North Africa and described them as *S. arabinotarda*, types A and B. Gober, Stacy, and Woodrow (1944) described a non-mannitol-fermenting strain, 8524, isolated from 47 persons in Mississippi over a 5-year period and found six additional times among cultures isolated in Louisiana and New York. It is apparent that mannitol-negative types other than *S. dysenteriae* and *S. ambigua* occur, and in fact they may be more common than either of these species (Gober *et al.*, 1944). Recently Berger (1945) described a new non-mannitol-fermenting dysentery organism, serologically related to the Flexner group, which he called *Bacterium wakefield*.

Identification of the mannitol-negative types, as of other enteric organisms, depends upon both biochemical and serological reactions. Because of the slight differences between certain types, such as some of those described by Large and Sankaran and by Sachs, and in view of the variation in the rate of arabinose fermentation by the strains of Christensen and Gowen, serological methods appear to provide the most reliable basis for strain differentiation. The purposes of this paper are to present a comparison of various mannitol-negative strains, to show serological methods for their identification, and to comment on their taxonomic position and on the importance of strain identification from an epidemiological viewpoint.

CULTURES AND METHODS

The strains of *S. dysenteriae* included old stock cultures as well as strains isolated from human infections as recently as 2 weeks before the tests were made.

The cultures of *S. ambigua* were both stock strains and recent isolates. Cultures of all the Sachs types were obtained from the Emergency Vaccine Laboratory (England); some of these types were also obtained from the Quetta Laboratory (India). Thirteen *S. arabinotarda* strains from Christensen and Gowen were received through the Army Medical School (Washington, D. C.); fifteen cultures of the 8524 strains from the Mississippi State Department of Health; three strains of *B. wakefield* from the National Collection of Type Cultures (England). In addition, cultures from various sources identified by us with one of these types have been included, as well as two cultures isolated in Mississippi which appear to constitute an additional serotype. Several other single strains having the biochemical characteristics of *Shigella*, but not identical with any of the other serotypes, are not included in the tables.

TABLE 1

Serological identity of Shigella strains Q771, arabinotarda type A, and 8524 as shown by reciprocal agglutination and adsorption tests

ANTISERUM	ADSORBED WITH	AGGLUTININ TITER WITH CULTURE		
		Q771	<i>S. arabinotarda</i> , type A	8524
Q771 (1909)		5,120*	5,120	5,120
	Q771	0†	0	0
	<i>S. arabinotarda</i> , A	0	0	0
	8524	0	0	0
<i>S. arabinotarda</i> type A (1973)		5,120	2,560	2,560
	Q771	0	0	0
	<i>S. arabinotarda</i> , A	0	0	0
	8524	0	0	0
8524 (1466)		5,120	5,120	5,120
	Q771	0	0	0
	<i>S. arabinotarda</i> , A	0	0	0
	8524	0	0	0

* Tube agglutination test after overnight incubation at 52 C.

† Titer less than 1:20.

Methods for preparing antisera, for performing slide and tube agglutination tests, and for making adsorption tests are the same as those previously described (Wheeler, 1944a, 1944b).

COMPARISON OF TYPES

Direct agglutination and reciprocal adsorption tests showed that strains of type Q771 (Sachs, 1943), of *S. arabinotarda* type A (Christensen and Gowen, 1944), and of type 8524 (Gober *et al.*, 1944) were identical. The results of recip-

rocal adsorptions of these three cultures are shown in table 1. Henceforth, these cultures, as well as strains of this serological type identified by us, will be referred to as type Q771, the designation used by Sachs and the culture number originally used by Large and Sankaran.

Strains of type Q1167 and *S. arabinotarda* type B were likewise found to be serologically identical. Cultures of this serotype also will be referred to by the Sachs designation, Q1167, until a standard nomenclature is accepted.

BIOCHEMICAL REACTIONS

The biochemical activity of the several mannitol-negative strains is shown in table 2, which is for the most part self-explanatory. Final readings of fermentation reactions were made at 28 days; urea utilization tests were done at 48 hours by the method of Rustigian and Stuart (1941); and trimethylamine oxide reduction tests were made after 24 hours' incubation as suggested by Weil and Black (1944).

All cultures failed to ferment mannitol and salicin and were Voges-Proskauer- and urea-negative. The other reactions are shown in table 2. A few points deserve mention. The cultures are divided into four groups: (1) indole-negative strains resembling *S. dysenteriae*; (2) indole-positive cultures (*S. ambigua* and similar types); (3) Sachs strains which differ from the description of the *Shigella* genus in one or more characteristics; and (4) a miscellaneous group consisting of a paracolon type and the organism described by Berger.

A majority of the *S. dysenteriae* strains (70 per cent) produced a weak acid reaction to bromcresol purple in lactose broth in from 14 to 28 days. These results were duplicated with three lots of lactose and with Berkefeld-sterilized as well as heat-sterilized media. All of the lactose-fermenting strains had been maintained on artificial media for a year or longer. Variation in the rate of fermentation of arabinose was noted with types Q771 and Q1167. Both of these have been described as slow fermenters (Christensen and Gowen, 1944), but about 20 per cent produced an acid reaction in bromcresol-purple arabinose broth in 24 hours. Repeated tests were not consistent as to the rate of fermentation.

The cultures in the second group resembled *S. ambigua* in giving positive indole reactions, and type Q902 could not be differentiated from *S. ambigua* by the other reactions listed. The new type, 1831, fermented xylose.

The organisms of the third group in table 2 differed from the description of the *Shigella* genus. All three produced gas from glucose; types B81 and B105 were motile, reduced trimethylamine oxide, fermented adonitol, and grew on Simmons citrate agar.

The last group of cultures in table 2 included a paracolon type which differed from normal *Escherichia* only in delayed fermentation of lactose, and three cultures of *B. wakefield* described by Berger (1945). When received, these three strains were nonmotile, anaerogenic, gram-negative rods having the characteristics of *Shigella*, except that all three gave good growth on Simmons citrate agar after 2 to 5 days of incubation. A weak acid reaction was produced in maltose after 22 to 29 days of incubation. Two strains were trimethylamine-positive.

The other was negative after 24 hours' incubation when first tested but was positive after 5 days' incubation; on subsequent tests this culture has been positive after 24 hours' incubation. Serial transfers in semisolid agar have yielded motile strains from all three cultures.

SEROLOGICAL REACTIONS

As is apparent from the biochemical reactions, type differentiation cannot be made with certainty without serological tests. Antiserums prepared from one or

TABLE 2
Biochemical reactions of mannitol-negative Shigella strains
(Negative as to mannitol, salicin, Voges-Proskauer, and urea)

CULTURE	NO. STRAINS	GLUCOSE	LACTOSE	SUCROSE	MALTOSE	RAMNOSE	ARABINOSE	DULCITOL	XYLOSE	SORBITOL	ADONITOL	CITRATE	INDOLE	T. M. A.	MOTILITY
<i>Shigella</i>															
<i>dysenteriae</i>	21	A	A*—	—	—	—	—	—	—	—	—	—	—	—	—
sp. Sachs Q771															
<i>arabinotarda</i> A															
sp. Gober 8524	26	A	—	—	A*—	—	AA*	—	—	AA*	—	—	—	—	—
sp. Sachs Q1167															
<i>arabinotarda</i> B	5	A	—	—	AA*	—	AA*	—	—	A—	—	—	—	—	—
sp. Sachs Q454	1	A	—	—	—	—	A	—	—	—	—	—	—	—	—
sp. Sachs Q1030	7	A	—	—	A*—	—	A	A*—	—	A*	—	—	—	—	—
<i>Shigella</i>															
<i>ambigua</i>	14	A	—	—	A*—	A	A*—	—	—	A*—	—	—	+	—	—
sp. Sachs Q902	2	A	—	—	A*	A	A	—	—	—	—	—	+	—	—
sp. 1831	2	A	—	—	A*	A	A	—	A	—	—	—	+	—	—
<i>Shigella</i>															
sp. Sachs A12	1	AG	—	—	A*	—	A	—	A*	A*	—	—	—	—	—
sp. Sachs B81	1	AG	—	A	—	—	—	—	—	—	A	+	+	+	+
sp. Sachs B105	3	AG	—	A	—	—	—	—	—	—	A	+	+	+	+
<i>Shigella</i>															
<i>paracolon</i> 2365*	2	AG	A*	—	A*	A	A	A	A	A*	—	—	+	+	+
<i>B. wakefield</i>	3	A	—	A*	A*	—	—	—	—	—	—	+	+	+	+

A = acid within 24 hours; A* = acid 24 hours to 28 days; AG = acid and gas within 24 hours; — = no fermentation.

* Mannitol was fermented by these strains.

more strains of each type have been tested with all serotypes. Relatively little cross reaction was found and that only in low titer. Type-specific reactions were readily obtained by the dilution method. Table 3 shows the specificity of slide agglutination tests with the diluted antiserums. Cross reactions were eliminated, and specific type determination was easily made with the unabsorbed serums.

Among the 14 *S. ambigua* strains examined, one, 1924,¹ was inagglutinable

¹ Received through Major K. S. Wilcox, AUS, at the Army Medical School from an overseas station.

in the slide test unless heated. This culture also differed from other *S. ambigua* strains in possessing only part of the *S. ambigua* antigen. Adsorption of *S. ambigua* antiserum with living, formalinized, or boiled culture of strain 1924 did not significantly reduce the homologous titer. On the other hand, the homologous agglutinins were completely removed from 1924 antiserum by stock *S. ambigua* strains.

One strain, 2164,² of type Q1030, isolated from a food handler, differed from other members of the mannitol-negative group in showing strong cross reactions with antisera for three other types (Q454, A902, Q1167) and slight reaction with another antiserum (B105). Adsorption with strain 2164 removed the homologous agglutinins only from Q1030 antiserum. After culture on agar for 8 months the strain lost all except the specific properties and reacted only with

TABLE 3
Agglutination reactions of mannitol-negative Shigella strains

CULTURE	ANTISERUM										
	<i>dysenteriae</i>	Q771	Q1167	Q454	Q1030	<i>ambigua</i>	Q902	1831	A12	B81	B105
<i>Shigella</i>											
<i>dysenteriae</i>	+	0	0	0	0	0	0	0	0	0	0
sp. Q771	0	+	0	0	0	0	0	0	0	0	0
sp. Q1167	0	0	+	0	0	0	0	0	0	0	0
sp. Q454	0	0	0	+	0	0	0	0	0	0	0
sp. Q1030	0	0	0	0	+	0	0	0	0	0	0
<i>Shigella</i>											
<i>ambigua</i>	0	0	0	0	0	+	0	0	0	0	0
sp. Q902	0	0	0	0	0	0	+	0	0	0	0
sp. 1831	0	0	0	0	0	0	0	+	0	0	0
<i>Shigella</i>											
sp. A12	0	0	0	0	0	0	0	0	+	0	0
sp. B81	0	0	0	0	0	0	0	0	0	+	0
sp. B105	0	0	0	0	0	0	0	0	0	0	+

Q1030 antiserum. Immune serum prepared from strain 2164 agglutinated only type Q1030 cultures.

Three coliform cultures isolated by Major W. H. Ewing were related serologically to type Q771. Two of these, one isolated from a food handler and one from a case of acute diarrhea, had somatic antigens identical with type Q771. Both were motile, aerogenic, slow-lactose-fermenting paracolon organisms, and the biochemical reactions were as shown in table 2 for paracolon strain 2365. The third strain differed in showing positive citrate and salicin, and negative dulcitol, reactions. This culture had part but not all of the Q771 somatic antigen. In an attempt to identify their flagellar antigens, these paracolon strains have been tested with sera prepared from 148 motile organisms cover-

² Received from Lieutenant Commander L. A. Barnes, USNR, Naval Medical School.

ing all sections of the coliform and paracolon group, *Proteus*, and some *Salmonella*. No flagellar reactions were obtained. Somatic agglutinations ranging in titer from 1:160 to 1:1,280 occurred with one *Proteus morganii*, two normal *Aerobacter*, and 2 paracolon *Aerobacter* serums. These strains possessed a Vi-like antigen present in Vi typhoid organisms and in certain coliform and paracolon cultures.

The three cultures received as *B. wakefield* showed no serological relationship to the mannitol-negative *Shigella*. Slight agglutination took place with certain Flexner group antisera (titers of 80 to 320). All three cultures agglutinated to 5,000 titer in a serum prepared against one culture having the biochemical reactions of *S. alkaescens* but lacking *S. alkaescens* antigens (Wheeler, Stuart, and Ewing, 1945, strain 2372). In the antiserum from another such strain, only slight agglutination occurred.

DISCUSSION

There are at least eight serotypes in the mannitol-negative section of the *Shigella* group. These include *S. dysenteriae*, *S. ambigua*, five of the Sachs types (Q771, Q1167, Q454, Q1030, Q902), and a new serotype (1831). All of these types comply with the generic description for *Shigella*, and each is serologically distinct. The inclusion in *Shigella* of the three Sachs types other than those mentioned is questionable.

Since Sachs type A12 produced gas from glucose, it probably should be excluded from the *Shigella* group unless it can be shown to be related serologically to recognized types or to produce anaerogenic variants. A somewhat similar situation in the paradysentery group occurs with the aerogenic Newcastle and Manchester varieties of the Boyd type 88. In this instance, however, the gas-producing Newcastle organism is serologically identical with other strains which in biochemical reactions are typical *S. paradysenteriae*. The variation in biochemical reactions of the Newcastle-Manchester-Boyd 88 group confuses the primary subdivision of the *Shigella* group on mannitol fermentation, since the Newcastle strains (Boyd, 1940) fail to ferment mannitol. In view of their antigenic composition and serological relationship to other races of *S. paradysenteriae* however, there is no question as to their classification in that species, and they are properly considered as biochemical varieties of *S. paradysenteriae* type VI. Possibly a serological relationship to other *Shigella* strains may be shown eventually for the A12 strain, in which case there will be justification for disregarding the aberrant biochemical tests.

Sachs B81 and B105 differ from other *Shigella* types in gas production and motility, and from the other mannitol-negative types in the trimethylamine reaction and in the fermentation of adonitol. It is true that motile variants of *Shigella* organisms have been described, such as motile *S. alkaescens* (Stuart, Rustigian, Zimmerman, and Corrigan, 1943), but here again the serological relationship to characteristic *Shigella* organisms is clear since the O antigens of the motile strains were identical with those of normal *S. alkaescens*. With the information at hand, types B81 and B105 are most conveniently classified with the paracolon bacteria.

Biochemical reactions of the *B. wakefield* cultures were similar to a paracolon type, 29911, described by Stuart, Wheeler, Rustigian, and Zimmerman (1943). When the cultures were tested with two antisera made from type 29911 strains, the motile variants of the *B. wakefield* cultures agglutinated to 5,120 titer in one of the sera. Because of these reactions and others to be discussed elsewhere (Stuart, Wheeler, and McGann, 1945), the *B. wakefield* cultures should be classified with the 29911 type of anaerogenic paracolon rather than in the *Shigella* group.

Other serological types undoubtedly will be added to the group. We have several single strains which cannot be classified in any of the serotypes discussed. Sachs (1943) likewise found several strains that he could not classify serologically, but many of these probably were *Proteus* types. Systematic study must await the collection of these strains. Here the value of a central reference laboratory for the comparison of strains is obvious. Already three independent descriptions have been made of the same organism (type Q771), in part because of the difficulties of obtaining strains for comparison.

The methods for identification of the types are relatively simple. Biochemical tests are satisfactory only for primary grouping. Although Sachs stated that the fermentation reactions of his strains were stable over a 3- to 4-year period, we have noticed two differences from his description: the Q1167 strains fermented arabinose, and some of the Q1030 strains failed to ferment dulcitol. The most reliable and simplest method of typing, once the organism is classified in the mannitol-negative group, is by agglutination with specific sera. A few cross reactions were observed. However, these were not due to major components and present no trouble in typing.

Cultures and antisera of the mannitol-negative group have been tested against antisera and cultures of the mannitol-positive group, the *Salmonella* group, and several members of the *Proteus* group. A number of reactions were obtained, usually of weak but sometimes of moderate strength. The antigenic components responsible for these reactions have not been identified.

One cross reaction of importance was between *S. dysenteriae* and *S. alkalescens*. Fourteen strains of *S. alkalescens* have been tested and found to agglutinate in seven of eight *S. dysenteriae* antisera to titers of 160 to 5120 (homologous titers 5,120 to 20,480), and to titers of 20 to 40 with the eighth serum. Certain coliform and paracolon cultures, serologically related to *S. alkalescens*, also were agglutinated to titers of 160 to 5,120. The reverse reactions of *S. dysenteriae* in *S. alkalescens*, coliform, and paracolon antisera were positive but were uniformly weak. Adsorptions failed to decrease the homologous titers significantly. The presence of these related antigens and the finding of paracolon cultures, such as strain 2365, with the complete somatic antigen of type Q771 furnish further evidence of antigenic continuity among members of the enteric group. Furthermore, the antigenic relationships shown for *S. dysenteriae*, its slow fermentation of lactose (particularly true of old stock cultures), and the finding, not only of carriers, but also of mild and even ambulatory cases of infection with this organism, indicate that the general textbook concept of *S.*

dysenteriae as an organism more highly specialized than other enteric bacteria is not valid.

The importance, from a laboratory standpoint, of recognizing the new serotypes is plain. Undoubtedly they have in many instances been confused with *S. dysenteriae* or *S. ambigua*, particularly when complete biochemical study or serological study could not be made. Some of these types, e.g., Q771, appear more commonly than *S. dysenteriae* or *S. ambigua*. For epidemiological work the need for typing is obvious. There is ample evidence for the pathogenicity of certain of the types, e.g., Q771, Q1167, and Q1030. Although most of the strains reported have been isolated from areas outside the United States, already we have had three of the new serotypes from persons resident in this country or recently returned from Africa and the Near East. Subclinical infection seems to be common, as reported by Gober *et al.* (1944) for type Q771. With the return of military personnel from endemic dysentery areas a greater incidence of all types, including the mannitol negatives, may be expected. The serological methods of type identification will be a practical aid in epidemiological investigations of *Shigella* infections.

Some comment is appropriate concerning the nomenclature of the mannitol-negative *Shigella* strains. The present designation by strain number is cumbersome. On the other hand, the species names proposed for certain types, e.g., *S. arabinotarda*, do not seem apt in view of the inconstancy of the characteristic described. Alternative and reasonably satisfactory designations suggest themselves, but, in order to avoid further complications of nomenclature, it seems advisable to refrain from proposing a scheme here.

SUMMARY

Type identification within the mannitol-negative section of the genus *Shigella* can be made readily by the slide agglutination test with diluted unadsorbed antisera.

A comparative study of mannitol-negative *Shigella* strains showed that those described as type Q771 (Sachs), *S. arabinotarda* type A, and Gober 8524 were serologically identical. Type Q1167 (Sachs) and *S. arabinotarda* type B were also identical but distinct from the others.

The existence of serological types among the mannitol-negative *Shigella* species was confirmed, and eight distinct serotypes were identified. These include *S. dysenteriae*, *S. ambigua*, five of the Sachs types, and one hitherto undescribed type. Three types described by Sachs (A12, B81, B105) were found to differ in biochemical or morphological (motility) properties from typical *Shigella* species and on the basis of information now available should not be included in the genus. A gas-producing, slow-lactose-fermenting, motile paracolon organism was described which had the identical somatic antigen of *Shigella* sp. type Q771 (Sachs). Three cultures which have been classified in the dysentery group as *B. wakefield* are more accurately classified in the anaerogenic paracolon group as the type 29911 of Stuart *et al.*

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DISSOCIATION IN *BRUCELLA ABORTUS*: A DEMONSTRATION OF THE RÔLE OF INHERENT AND ENVIRONMENTAL FACTORS IN BACTERIAL VARIATION

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Brucella abortus exhibits the phenomenon of dissociation, that is, changes in colony form, culture characteristics, cell morphology, immunological reactions, biochemical reactions, and virulence. Henry (1933) has presented a detailed description of dissociation in the genus *Brucella*, which usually involves changes from the antigenically active smooth (S) type to intermediate (I), and to antigenically inactive rough (R), brown (Br), and other types.

This type of dissociation resembles variations which are common in many bacterial species. Hadley (1927, 1937) has published general reviews of bacterial dissociation and interpreted such changes as due to normal cyclogenic development. Others (e.g., Mayer, 1938; Reed, 1940; Dubos, 1945) have tried to explain the changes which occur during dissociation as being due to spontaneous hereditary changes (mutations), with a subsequent selection of mutants which can best persist in any given environment. Many investigators (e.g., Hinshelwood and Lodge, 1944) have postulated a direct influence of the environment upon such spontaneous changes. Criticism of Hadley's "ontogenetic" theory has arisen from experimental work which has demonstrated the lack of linked character variation, such as agglutinative behavior and cell form, in the change from the S type to the R type (Humphries, 1944). Such criticism is further substantiated by recent work which provided substantial proof for the existence of undirected, spontaneous hereditary changes (mutations) in bacteria (Demerec, 1945; Demerec and Fano, 1945; Anderson, 1944; Luria and Delbrück, 1943; Gray and Tatum, 1944; Roepke, Libby, and Small, 1944; Severens and Tanner, 1945).

Whereas the last-mentioned investigators focused their attention mainly on the mutational step per se, another line of approach to the problem of bacterial variation was provided by studies on differences between dissociating populations (clones) started from single cells and maintained under standard conditions. Thus Braun (1945), in a preliminary report on factors controlling bacterial dissociation, furnished data demonstrating the existence of inherent differences between clones in regard to dissociation percentages,² evidence suggestive

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² Previously, in describing methods for the comparative estimation of the percentage of dissociated cells within a population after 10 days of growth in broth, the name "dissociation rate" was suggested for the percentage of dissociation observed (Braun, 1945). Since the term "rate" may be misleading in this connection, it is now proposed to use the term "dissociation percentage" generally for the amount of dissociation observed after various periods of growth, and to call the dissociation percentage in 10-day-old broth cultures the "dissociation index" of a given strain.

of spontaneous appearance of dissociated types and their subsequent establishment within a population under the control of inherent and environmental factors. A more detailed account of the work which led to the detection of these inherent factors and an evaluation of their role in controlling dissociation will be presented here. A number of environmental effects superimposed upon the action of these inherent factors will be described and an interpretation of dissociation in terms of these results will be attempted. The order in which the data were actually obtained will be used as the order of presentation.

MATERIAL AND METHODS

The strains used throughout this work originated from *Brucella abortus*, strain 19, cultures, which have been periodically distributed by the United States Bureau of Animal Industry. They are numbered 19-1, 19-2, 19-3, etc., according to the date of their distribution. The strain most extensively used in the present work, namely, 19-9, was received at this station on October 26, 1942; it was kept on potato agar slants and was periodically transferred and checked for purity. A rough strain was isolated from old saline suspensions of 19-9 S early in 1943. This strain, called 19-9 R, was also kept on potato agar slants and was transferred and checked for purity periodically. To date, no dissociation has occurred in these stock cultures.

The percentage of dissociation (see footnote 2) was tested by suspending morphologically identical colonies in beef infusion broth at 37 C. During the early experiments an arbitrary number of colonies was suspended in a small amount of saline, and equal amounts of this suspension were inoculated into each tube of broth. Later on one picked colony was inoculated into each tube of broth, or one picked colony was suspended in saline. The suspensions were then adjusted according to density, and equal amounts of these adjusted suspensions were inoculated into the broth. The amount of broth in each tube was generally adjusted to 5 ml. Platings were made at various times after the start of the broth cultures. One loop of broth was streaked on 2 per cent glycerol, 1 per cent glucose agar plates; the plates were incubated at 37 C for four days and then checked for dissociated colonies under the low power of a dissecting microscope with an obliquely transmitted lighting arrangement (Henry, 1933). If dissociated colonies were present, at least 100 colonies of each plate were counted and classified in the region where colonies were well separated; and the percentage of dissociated colonies was estimated from such counts. Although the presence of *different* types of dissociated colonies was roughly estimated, no attempt was made actually to count the relative number of rough (R), brown (Br), intermediate (I), and other types among the dissociated colonies. That is, if a broth culture had been started with smooth colonies, the percentage of all nonsmooth colonies on the test plate was determined. If a broth culture had been started with rough colonies, the percentage of all nonrough colonies was counted.

When buffered broth was used the broth was prepared with McIlvaine's standard buffer solutions instead of water. The pH was usually tested with the

help of indicators, except in certain experiments in which small changes of pH were checked with a Coleman pH meter.

The isolation of single cells was performed according to the method of Johnstone (1943). This method proved to be entirely satisfactory once adequate experience had been gained in identifying single cells on the agar surface. The most important factor in this identification is correct lighting and use of the substage optical system.

Viability counts were made by the appropriate dilution of culture samples and the counting of colonies which grew on plates with 8 per cent cooked horse blood agar (chocolate medium) after the plates had been streaked with 0.1 ml. of the diluted sample. Total counts were made on samples from appropriately diluted cultures with the help of a Petroff-Hausser counting chamber and dark-field illumination at a magnification of 320 \times .

TABLE 1

Percentage of dissociated colonies on plates made at various intervals after start of broth cultures with different pH

pH OF BROTH	BROTH INOCULATED WITH	PERCENTAGE OF DISSOCIATED COLONIES AFTER					
		2 days	6 days	13 days	17 days	26 days	35 days
6.6	S	none	1	10	50	50	50
	R	1	5	5	5	10	no growth
7.2	S	none	1	1	2	2	2
	R	none	none	none	none	none	none
8.6	S	none	none	none	none	none	none
	R	none	none	none	none	none	none

RESULTS

Effect of the pH of Broth on Dissociation

The differential effect of broth with different pH values on dissociation was first noted when one batch of broth was accidentally made too alkaline (pH 8.6). When S or R type organisms were suspended in this alkaline broth, no dissociation occurred for as long as 35 days after the broth cultures were started, whereas normally dissociation became apparent a few days after the start of a broth culture. Subsequently, two other batches of broth were prepared, one with a pH of 7.2 and one with a pH of 6.6. Suspensions (Gates 4) were made of 19-9 S and of 19-9 R, and 0.1 ml of either the S or the R suspensions was added to 10 ml of broth. Plates were made from these broth cultures at frequent intervals after inoculation. The resulting (estimated) percentages of dissociation, in a series of tests for each batch of broth, are summarized in table 1. These tests showed that slight acidity of the media (pH 6.6) favored dissociation, whereas little dissociation occurred at pH 7.2 and none at pH 8.6.

In another set of experiments the effect of the pH of the media on dissociation

was further confirmed. This time 1 ml. of buffer solution of a definite pH (McIlvaine's standard buffer solution) was added to 2 ml of unbuffered broth. The addition of solutions of pH 3 and pH 4 inhibited growth of both S and R types. Considerable dissociation was observed in tubes to which a buffer solution of pH 5 had been added, much less in tubes to which solutions of pH 6 and pH 7 had been added, and none when a buffer solution of pH 8 was added.

On periodical inspection of changes in the pH of unbuffered broth after inoculation with S type cells, it was found that active growth of the bacteria increases the pH. The pH of 20 tubes of unbuffered broth was 6.8 on the day of inoculation; 2 days later all tubes showed a pH of 7.4; 4 days after inoculation the pH was 7.6; and it was 8.0 when tested on the sixth day. It remained at 8.0 until the cultures were discarded 2 weeks later. Regardless of whether

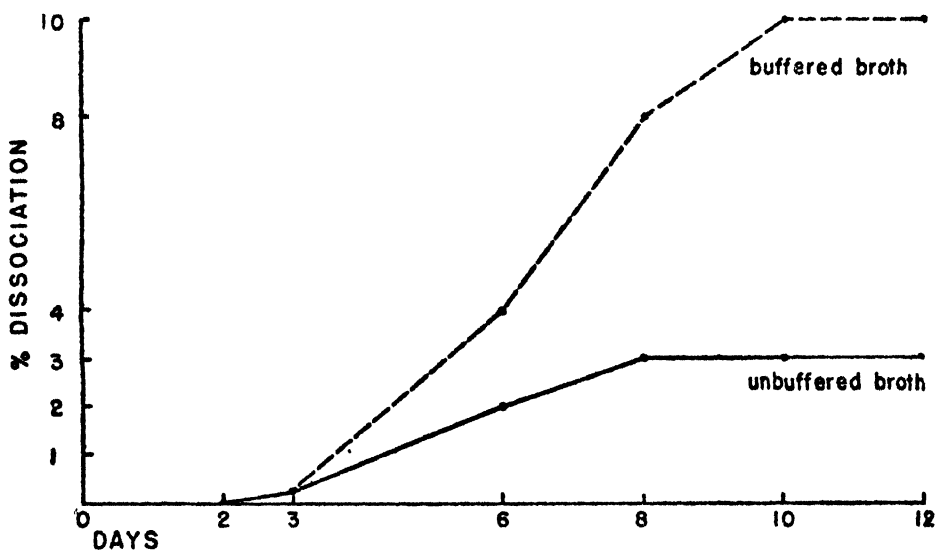


FIG. 1. PERCENTAGES OF DISSOCIATION OF IDENTICAL STRAINS AFTER VARYING PERIODS IN BUFFERED OR UNBUFFERED BROTH

the initial pH was 6.8, 7.0, 7.2, or 7.4 in subsequent tests, the pH of the unbuffered broth always increased to 8.0 a few days after inoculation with *Brucella abortus*.

The change in daily percentage of dissociation parallels this change of the pH in unbuffered broth, i.e., dissociation proceeds more rapidly during the period when the pH is low and reaches an equilibrium when the pH of the medium approaches its 8.0 equilibrium (figure 1). Similar tests with buffered broth later revealed that, under conditions of constant pH, dissociation percentages increase at a more constant rate, reaching a maximum point of dissociation after about 10 days (figure 1).

Since it was known that the optimum pH for the growth of *Brucella abortus* is 6.8 (Huddleson, 1943), a relationship between growth rates and percentage

of dissociation was immediately suspected at this point, and this was later experimentally confirmed (table 7).

Whereas these results provided proof concerning the effect of the pH of the environment upon dissociation percentages, further tests, utilizing different strains, soon indicated that the pH acts only as a modifying, environmental factor on dissociation percentages, whereas other factors, presumably inherent, determine the potential range of dissociation percentages for each strain. This was first recognized when three different smooth strains were subjected to growth in broth of identical pH. All three strains originated from the 19-9 S

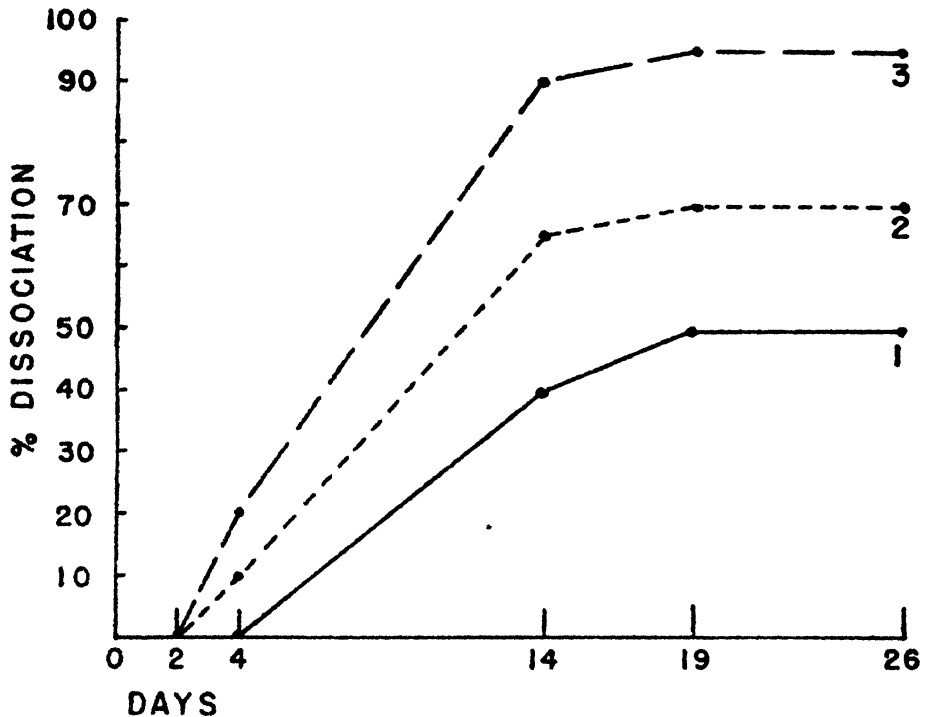


FIG. 2. PERCENTAGES OF DISSOCIATION OF THREE SMOOTH STRAINS AFTER VARYING PERIODS IN BROTH

culture. Strain 1 represents the original 19-9 S culture, i.e., an S type which has been stable for at least 1 year. Strain 2 represents an S type which was isolated from an experimentally produced I type which had reverted to S 6 months previously, and strain 3 had an origin similar to strain 2 but had reverted to S only 2 months previously. Suspensions of equal density of each of these three morphologically similar strains were inoculated into broth with a pH of 6.8, and the percentage of dissociated cells was ascertained 2, 4, 14, 19, and 26 days after inoculation. The results are represented graphically in figure 2. Although growing in identical environments, the three morphologically identical strains

showed clearly different dissociation percentages: strain 3 showed the highest dissociation percentages during all tests, strain 2 was characterized by consistently lower dissociation percentages, and strain 1 exhibited not only the lowest percentages of dissociation but also a later onset of dissociation.

In another test, one S strain showed 50 per cent dissociation after 10 days of growth in buffered broth of pH 6.6 and 2 per cent dissociation after 10 days in buffered broth of pH 7.4, but another morphologically similar strain showed 1 per cent dissociation after 10 days at pH 6.6 and none at pH 7.4.

Like results were obtained with two morphologically similar rough strains, both of which had been obtained by dissociation of 19-9 S after prolonged growth in broth, but they had been isolated at different times. Table 2 presents the results of two different tests, one with unbuffered broth with an initial pH of 7.2 and one with unbuffered broth of pH 6.8. Aside from illustrating the constant differences in percentage of dissociation between the two strains at pH 7.2 as well as pH 6.8, these results again demonstrate the differences which are

TABLE 2

Percentage of dissociated colonies on plates made at various intervals after start of broth cultures from two rough strains; illustrating the effects of strain differences and of the pH of the environment upon degree and onset of dissociation

pH OF BROTH	R STRAIN USED	PERCENTAGE OF DISSOCIATED COLONIES AFTER								
		2 days	4 days	5 days	7 days	8 days	12 days	15 days	17 days	25 days
6.8	1		none		none		none			
	2		none		none		20			
7.2	1	none		none		none		none	none	30
	2	none		none		none		none	none	70

found in onset and percentage of dissociation if broths of different pH are used, regardless of the potential tendency for dissociation of any given strain.

Inherent Factors Controlling Percentages of Dissociation

In order to obtain more information on those apparently inherent factors which determine the potential range of percentage of dissociation of a strain, it was first necessary to establish a standard set of conditions in which the degree of dissociation of different cultures could be compared, since it had been shown that dissociation percentages were subject to modifications by changing environmental conditions such as pH. Buffered broth of pH 6.8 was chosen as the standard experimental environment, and the percentage of dissociation on the tenth day of growth in this broth (each tube inoculated with one colony) will be called the "dissociation index" of a strain, i.e., the percentage of dissociated colonies observed on plates made from 10-day-old broth cultures.

A convincing proof for the existence of inherent factors controlling dissociation percentages was obtained by the utilization of a new technique of single cell isolation, first described by Johnstone (1943). The following procedure

was used: A single cell was isolated on an agar-covered slide, and, after its growth into a colony, cells from this colony were streaked on a 2-1 agar plate. Colonies arising from these daughter cells of a single isolated cell were then picked, and each colony was suspended individually in a tube of broth. After 10 days, samples from each broth culture were streaked on 2-1 agar plates, and the plates were checked for percentages of dissociated colonics 4 days later.

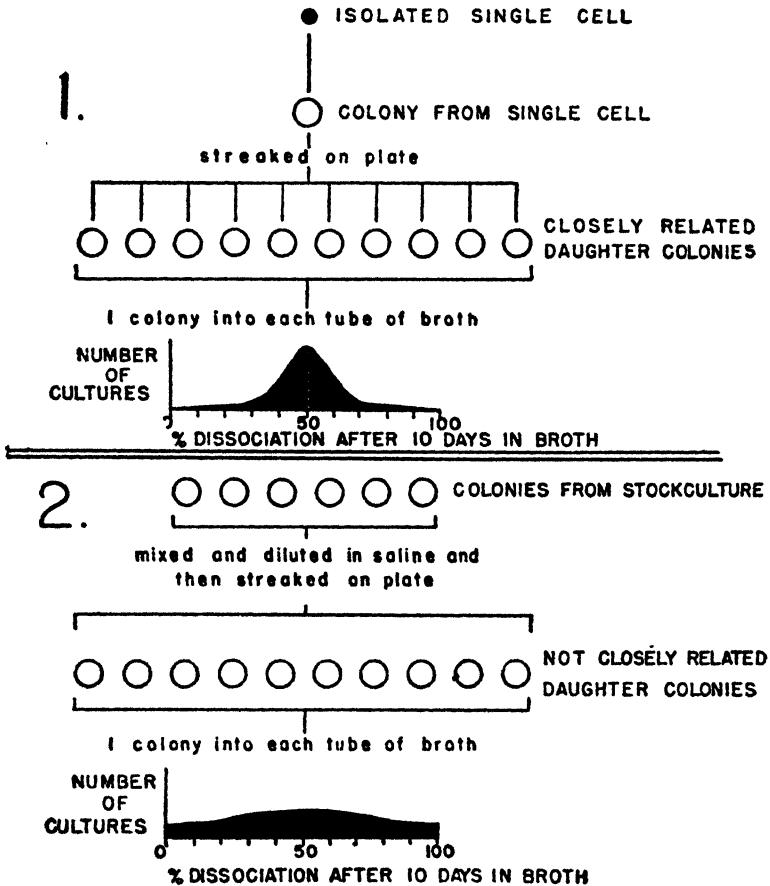


FIG. 3. DIAGRAMS ILLUSTRATING THE METHOD USED IN DEMONSTRATING THE EXISTENCE OF INHERENT FACTORS CONTROLLING DISSOCIATION PERCENTAGES

For further details see text

Since the individual colonies originated from a single cell, the dissociation indices observed after growth in each of the broth cultures were expected to be fairly close to a mean if the inherent dissociation potential is greater than the variability (mutability) of an individual cell. This is illustrated in diagram 1 of figure 3.

At the same time a similar test was made on colonies which, although from the same culture, originated from various single cells. As illustrated in diagram

2 of figure 3, a considerable number of colonies were taken from the same stock culture from which the single cell used in the first part of this experiment originated. The colonies were suspended in saline and mixed, and the diluted suspension was then streaked on a 2-1 agar plate. Again, isolated colonies were picked and individually suspended in a tube of broth. The rest of the experiment was exactly like that in the first part: after 10 days samples of the

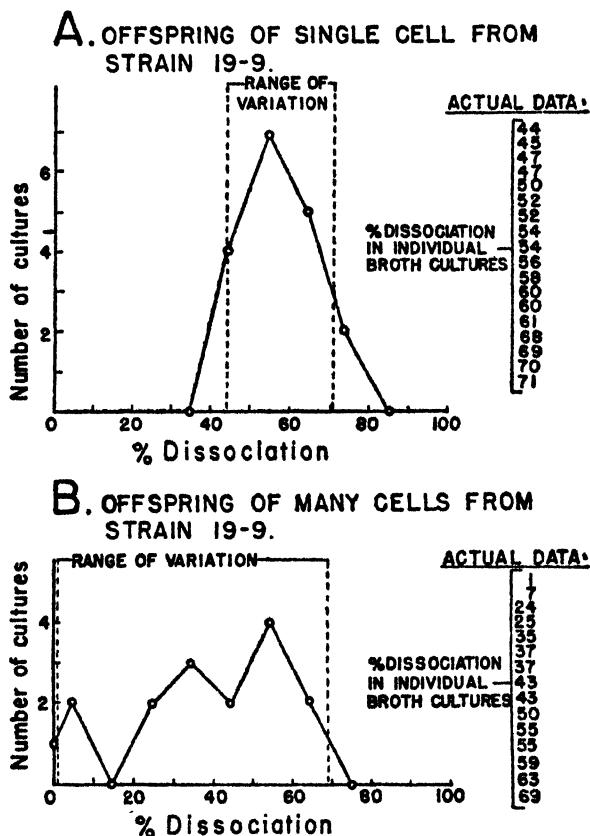


FIG. 4. A DEMONSTRATION OF THE EXISTENCE OF INHERENT FACTORS CONTROLLING DISSOCIATION PERCENTAGES

Actual results obtained in experiments conducted according to the method illustrated in figure 3.

broth cultures were streaked on plates, and the percentage of dissociated colonies was recorded 4 days later. If differences in inherent factors controlling dissociation percentages exist between cells of different origin and if such potentials are greater than the variability (mutability) of each cell, then the dissociation indices observed in this second experiment would be expected to be more widely scattered around a mean than those observed in the first experiment in which colonies originated from one single cell. This is graphically illustrated in diagram 2 of figure 3.

The actual results followed the expectations just expressed, supporting the idea that inherent factors control dissociation percentages. Figure 4A illustrates the results of an experiment which was started from a single cell according to diagram 1 of figure 3. The dissociation indices of 18 broth cultures, started from closely related colonies, show a distinct mean around 55 per cent, with relatively little variation extending to 44 per cent and 71 per cent. Considerable variation from a mean could be observed in the control experiment, which was

TABLE 3
Examples of dissociation indices in various clones (B-F)

	A	B	C	D	E	F
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
	1	44	74	28	7	0.0
	7	45	76	32	7	0.0
	24	47	78	36	7	0.0
	25	47	80	36	7	0.3
	35	50	81	41	9	3
	87	52	82	42	9	3
	87	52	87	43	9	4
	43	54	88	44	13	5
	44	54	91		14	13
	50	56	95		15	
	55	56	95			
	55	60				
	59	60				
	63	61				
	69	68				
Dissociation constant*	40 ± 5.09	54 ± 1.74	84 ± 2.23	38 ± 1.99	10 ± 0.95	3 ± 1.27
Number of colonies counted...	1,640	1,586	1,777	1,220	1,687	1,929

A. Dissociation indices of individual colonies originating from many single cells isolated from strain 19-9.

B. Dissociation indices of individual colonies originating from one single cell isolated from strain 19-9.

C-F. Examples of dissociation indices of colonies from strains started from single cells.

* Dissociation Constant = most representative dissociation index of a population = \bar{p} of Hendricks, *Poul. Science*, 14: 365.

conducted according to diagram 2 of figure 3 and the results of which are presented in figure 4B. The graph illustrates clearly the irregular dispersion of dissociation indices in this less related material, the percentages of dissociation varying from 1 per cent to 69 per cent.

Subsequently, progenies from many other single cells, isolated from various strains, were tested, and the similarity of dissociation indices within clones was confirmed. A few representative samples are given in table 3 together with the

results of a statistical analysis which proved that differences in dissociation indices between clones are statistically highly significant. The clones with different dissociation indices were obtained by systematic selection. This means that single cells from existent strains were tested for dissociation indices, and the heterogeneity or homogeneity of various strains were thus established (examples of four B.A.I. strains are presented in table 4). If a strain showed a fairly high percentage of low dissociating cells, attempts were made to establish low dissociating clones from that strain (for example, see clone D of table 3, which was obtained from strain 19-12 of table 4). Correspondingly, high dissociating clones were obtained from strains for which preliminary tests had

TABLE 4
Examples of dissociation indices of colonies from four B.A.I. strains

STRAIN	TOTAL NUMBER OF COLONIES COUNTED	DISSOCIATION INDICES OF COLONIES	MEAN
		<i>per cent</i>	<i>per cent</i>
19-9	1,640	See B in fig. 4	54
19-11	952	21 29 33 46 78	41
19-12	842	22 40 52 81 96	58
19-13	990	70 77 79 95 96	83

TABLE 5
Dissociation indices of colonies from a heterogeneous strain illustrated by three broth cultures from each colony

		PARENT COLONY									
		A	B	C	D	E	F	G	H	I	K
Dissociation indices (%) in 3	(1)	5	5	5	28	24	37	33	36	52	4
broth cultures	(2)	3	8	11	19	40	20	32	49	45	4
from each colony	(3)	2	8	6	25	24	35	56	49	68	62 (1)
Total number of colonies counted		544	509	531	517	435	471	430	465	459	462

indicated the presence of high dissociating cells. A survey of available strains proved that many of them are highly heterogeneous in regard to the dissociation index, whereas only a few are fairly homogeneous. Selected clones have so far shown great stability in regard to retention of their original dissociation index if preserved on agar slants at low temperatures.

Additional proof for the genetic control of dissociation percentages was obtained by the following test: Single colonies, picked from a plate made from a heterogeneous stock culture, were individually suspended in 1 ml of saline, and 3 broth cultures were then inoculated with equal suspensions (0.3 ml) of the same colony. The results, compiled in table 5, show that generally the dissociation indices of cultures from the same colony are very much alike, but

dissociation indices differ considerably between colonies. The occasional lack of agreement between cultures made from one colony of this heterogeneous population is to be expected, since colonies do not always arise from one cell only. Furthermore, in this and all other experiments on dissociation percentages in clones, a small percentage of spontaneous changes in inherent factors controlling dissociation percentages has to be taken into consideration. Future experiments are expected to yield information on the actual frequency of such changes.

Environmental Factors, Other than pH, Affecting Dissociation Percentages

After clones with known dissociation indices under standard conditions had been established, it became possible to test the modifying effects of a number of environmental factors upon genetically controlled dissociation percentages.

Daily transfer. An S clone which showed 45 per cent dissociation after remaining for 8 days in the same tube of broth (buffered pH 6.8) was used for a test in which 0.1 ml of a saline suspension of this strain was inoculated: (a) into a number of broth cultures which were not disturbed for 8 days, and (b) into a number of identical broth cultures of the same batch of broth, which did not remain undisturbed for 8 days, because 0.5 ml of 1-day-old broth cultures were daily transferred into fresh tubes of broth.

When plates were made on the eighth day from the 8-day-old undisturbed cultures of (a), 45 per cent dissociation was recorded; whereas plates made from the seventh transfer of (b), i.e., organisms which had grown for 8 days in daily renewed broth, showed less than 1 per cent dissociation. It was thought that this difference in dissociation percentages between growth in aging broth and growth in daily renewed broth might be due to one or more of a combination of three factors: (1) Actively growing (multiplying) cells may produce a metabolic product which enhances dissociation, and the effect of such metabolites would be lessened in case of daily transfers. (2) The growth of organisms may produce a deficiency in the broth which enhances dissociation. Again daily transfers would almost nullify the effect of such a deficiency. (3) Since, in comparison with aging broth cultures, daily transferred cultures have a smaller size of population per ml of broth, a lack of population pressure may be responsible for the failure of dissociated types to establish themselves within the daily transferred population. This infers that population pressure has a major role in the establishment of dissociated types and suggests that differences in population pressure may affect the propagation and survival of S types and dissociated types differentially. In daily renewed broth dissociated types may then have less of a chance to establish themselves because of the lack, or low intensity, of population pressure; but in aging cultures, in which the struggle for survival of different types can be assumed to be much more intense, a high degree of population pressure exists, favoring the establishment of dissociated types.

It was possible to eliminate the first two possibilities, (1) and (2), as responsible factors through the following test: S type organisms were suspended in filtrates

of broth in which other S type organisms of *Brucella* had grown and dissociated previously for 2 weeks. Daily transfers to tubes containing 5 ml of filtrate only and to tubes containing 3 ml of filtrate plus 2 ml of fresh broth were then made. In another set the organisms remained for 10 days without transfer in tubes with filtrate only or in tubes with filtrate plus fresh broth. Controls consisted of tubes with fresh broth only and tubes with 3 ml fresh broth plus 2 ml saline. Daily inspection of the percentage of dissociation did not reveal any differences between controls and tubes containing filtrate. After the possibility had thus been eliminated that metabolite or deficiency factors caused the striking differences in dissociation percentages between growth in aging broth and daily renewed broth, the role of population pressure in dissociation came into the foreground of attention.

Different batches of broth. The next suggestive evidence regarding an effect of population size on dissociation percentages was obtained when it was observed that certain batches of broth yielded different dissociation indices when cells from identical clones were used (see example in table 6). Although the proportion of ingredients was the same in all batches of broth, the peptone and beef

TABLE 6
Effect of different batches of broth upon dissociation index (D. I.) and growth
(Averages for 21 cultures)

BROTH BATCH	D. I. OF NO. 1287	D. I. OF NO. 1863	D. I. OF NO. 1983	TOTAL COUNT PER ML AFTER 10 DAYS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>billions</i>
IX	0	1	0	1.375
X	13	7	5	2.475

extract used did not originate from the same lot. Differences in nutrient material supporting growth were, therefore, suspected, and an inspection at different times of the total number of bacteria in two such batches of broth, originally inoculated with an equal number of cells, revealed such growth differences. Table 6 illustrates the higher dissociation percentages observed in broth which supported better growth.

Daily plating versus one plating, and the effect of temperature changes. Another striking effect of modified growth rates upon dissociation percentages of cells from the same clone was observed in a large number of experiments, which were conducted as follows: Tubes with broth (from the same batch of broth) were inoculated with equal amounts of a suspension made from cells belonging to one clone. One set of tubes was disturbed daily when samples were removed for streaking on plates; another set of tubes was left undisturbed for 10 days. Table 7 shows a representative example of the results obtained.

The dissociation index in a daily disturbed population is much lower than the dissociation index of the genetically identical population left undisturbed for 10 days. Counts revealed that the total number of cells after daily disturbance is smaller than the number of cells in equally old but previously undisturbed

cultures. Again, less growth produced a lower dissociation index. The growth differences between "disturbed" and "undisturbed" cultures are probably caused

TABLE 7

Dissociation percentages and total count in buffered and unbuffered broth cultures from one clone after frequent plating vs. one plating on 10th day only

(Averages for 32 cultures)

Broth	DISSOCIATION PERCENTAGE ON VARIOUS DAYS AFTER START OF BROTH CULTURES					TOTAL COUNT PER ML ON 10TH DAY
	2	4	6	8	10 <i>per cent</i>	
Buffered	None	None	None	None	0.1	1.2 billions
Buffered .. (Plated 10th day only)	None	None	None	None	11	1.75 billions
Unbuffered	None	None	None	None	None	550 millions
Unbuffered .. (Plated 10th day only)	None	None	None	None	0.01	700 millions

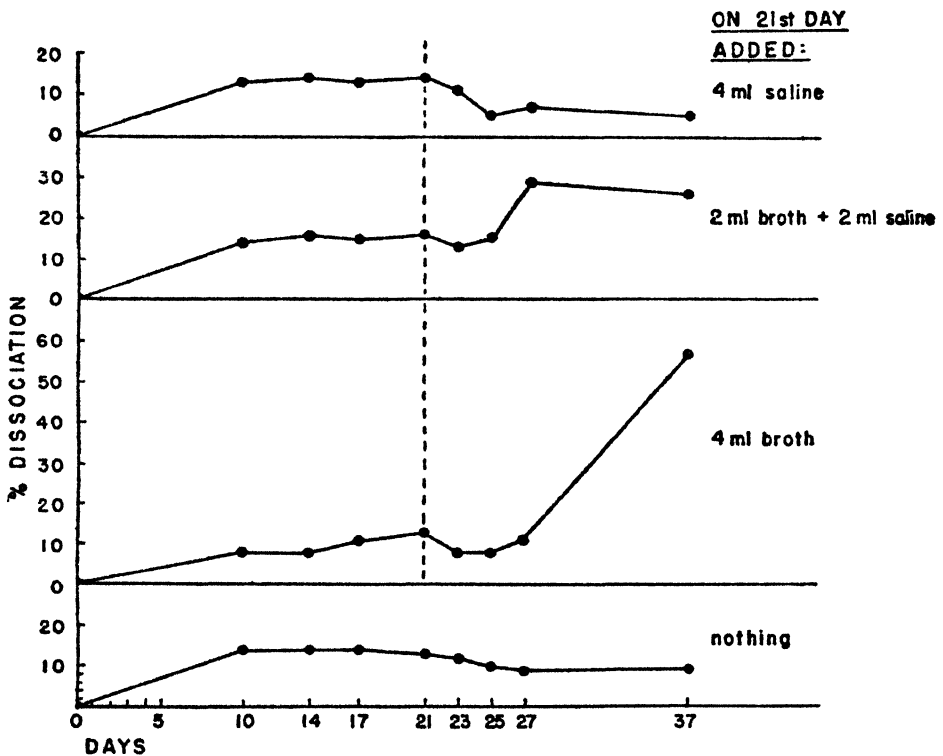


FIG. 5. THE EFFECT OF EXHAUSTED VERSUS RENEWED BROTH UPON DISSOCIATION PERCENTAGES

by temperature changes to which the "disturbed" cultures were subjected when they were daily removed from the incubator for streaking. It was subse-

quently established that temperature differences have a pronounced effect on growth rate, viability, and dissociation.

Exhausted versus renewed broth. The dependency of dissociation upon growth was next proved by adding fresh broth to aged broth cultures which had ceased to support growth. It had previously been observed that after about 10 days of growth in broth dissociation percentages reached an equilibrium (see figure 1), and counts of the total number of cells later showed that growth actually ceases at this point. Therefore, a number of broth cultures were now started with identical inocula; dissociation percentages were then checked on the 10th, the 14th, the 17th, and the 21st days. To four of the 21-day-old broth cultures 4 ml of fresh broth were added; to two others 2 ml of fresh broth and 2 ml of saline were added; to four others 4 ml of saline were added; and two cultures were left undisturbed. Dissociation percentages were then checked 2, 4, 6, and 16 days later. Representative results are presented in figure 5. It can be observed that dissociation percentages, after having reached an equilibrium in exhausted broth, rise significantly if fresh broth is added, i.e., if new growth and subsequent competition is initiated.

TABLE 8

The effect of reduced O/R potential of broth upon dissociation index (D. I.), growth, and viability

	D. I. OF NO. 1287 (AVERAGE OF 16 CULTURES)	TOTAL COUNT PER ML ON 14TH DAY	VIABLE COUNT PER ML ON 14TH DAY
	<i>per cent</i>	<i>millions</i>	<i>millions</i>
Normal Broth	13	980	620
O/R Broth	less than 1	700	500

Reduced O/R potential. The most striking demonstration of the effect of growth rates upon the establishment of dissociated types within a population was obtained when broth with a lowered oxidation-reduction potential was used. Such broth was prepared by the addition of 0.1 per cent agar and 0.1 per cent sodium thioglycolate to standard broth (Reed and Orr, 1943). Eight tubes of standard broth and eight tubes of "O/R broth" were each then inoculated with 0.2 ml of the same suspension of bacteria. Plates made from these cultures indicated a considerable percentage of dissociated cells in samples from the standard broth, but less than 0.1 per cent of dissociated cells were found in cultures made with "O/R broth" (table 8). Counts made on the fourteenth day of the total number of cells and the number of viable cells revealed a far smaller number of both per ml of "O/R broth" than per ml of standard broth (table 8). Thus it was found again that environmental conditions which alter growth rates and affect viability cause a striking modification of genetically controlled dissociation percentages.

Growth Rates and Dissociation Percentages

The results described above provided a great deal of indirect evidence on the major role of growth rates and population pressure in the determination of

dissociation percentages. The necessary direct evidence was finally supplied by actual counts of bacteria.

Before the results of such counts are reported, some general principles of growth in bacterial populations may be stated. Jordan and Jacobs (1944) reported on observations with *Bacterium coli* which showed that as long as food is supplied the total number of organisms increases steadily, but the number of viable cells soon reaches an equilibrium. In the present work with *Brucella abortus* these observations were confirmed (see figure 6). Furthermore, it was

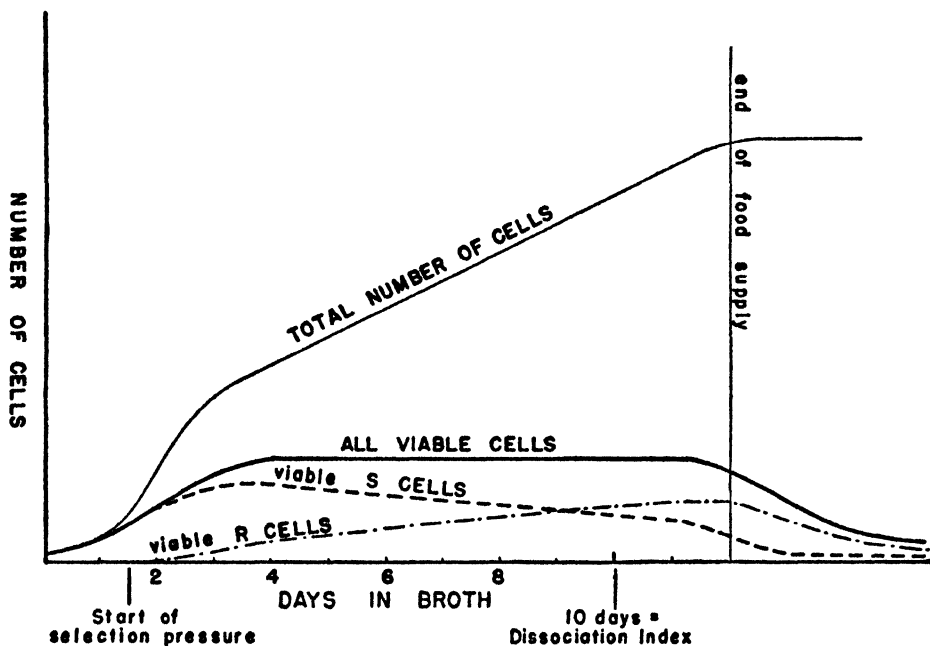


FIG. 6. A GRAPHICAL REPRESENTATION OF THE TOTAL NUMBER OF CELLS AND OF THE NUMBER OF VIABLE CELLS DURING GROWTH IN BACTERIAL POPULATIONS AND AFTER CESSATION OF GROWTH DUE TO EXHAUSTION OF FOOD SUPPLY

The curves of "total numbers of cells" and "all viable cells" are based on experimental results by Jordan and Jacobs (1944) with *Bacterium coli*, which have been substantiated in the studies with *Brucella abortus* reported here. For an explanation of the curves of "viable S cells" and "viable R cells" see discussion.

determined that a maximum exists for the number of viable cells which can be found per ml of broth (approximately 500 millions per ml with possible slight variations between clones). Regardless of the size of the inoculum this maximum is always reached within the first 4 days after the start of growth in broth and is retained as long as growth persists. If a culture is started with more than 500 million per ml of viable cells, the number of viable cells will decrease to the "maximum level" of around 500 millions per ml within 24 hours and remain at that level as long as growth persists (figure 7).

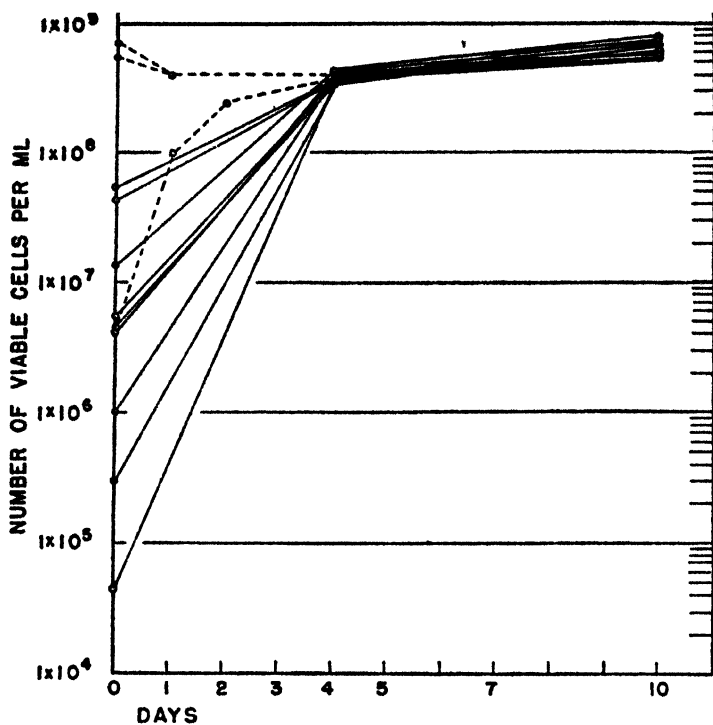


FIG. 7. NUMBER OF VIABLE CELLS PER ML IN BROTH CULTURES STARTED WITH VARYING AMOUNTS OF INOCULA, ILLUSTRATING THE LIMITS FOR VIABLE CELLS PER ML OF BROTH REGARDLESS OF THE AMOUNT OF THE INOCULUM

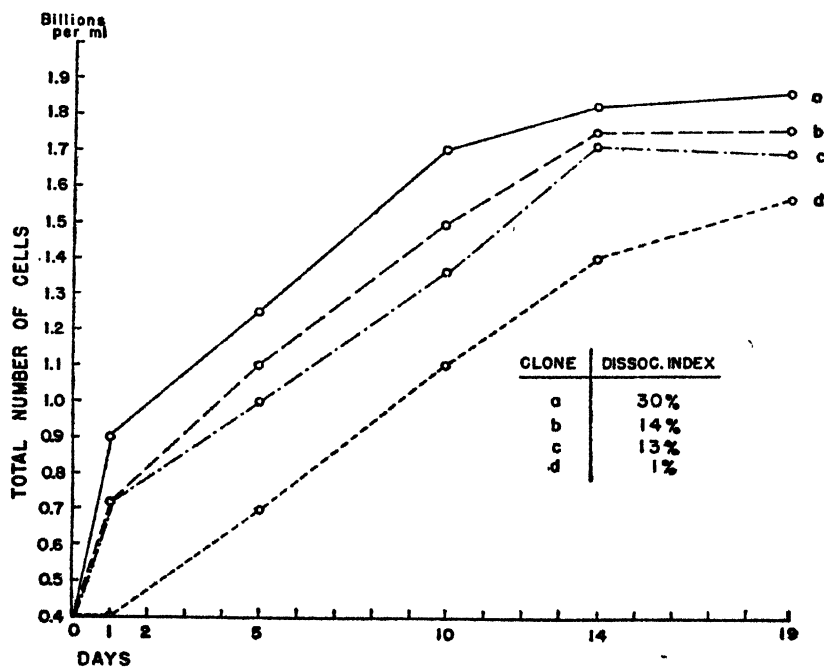


FIG. 8. GROWTH RATES OF FOUR CLONES WITH DIFFERENT DISSOCIATION INDICES

The total number of cells per ml have been adjusted to compensate for evaporation of the broth.

An effect of the size of the inoculum upon dissociation percentages has been observed and can be explained in terms of differential initial population pressure due to the limits for number of viable cells per ml. In the majority of tests, broth cultures with larger inocula (and thus presumably greater initial competition) showed higher dissociation percentages than cultures of the same clone inoculated with a smaller number of organisms. A representative example is given in table 10.

The total number of cells and the number of viable cells in growing populations of several clones were determined in broth cultures which had been started in duplicate with equally large inocula. The results which are presented in figure

TABLE 9

Average of total number of cells and number of viable cells in smooth and rough cultures, respectively, after 7 and 10 days of growth in broth

		DAYS AFTER START OF CULTURES	
		7	10
Smooth	Total count per ml	0.85×10^9	2.10×10^9
	Viable count per ml	0.49×10^9	0.55×10^9
Rough	Total count per ml	0.54×10^9	1.00×10^9
	Viable count per ml	0.54×10^9	0.54×10^9

TABLE 10

The effect of the size of the inoculum upon dissociation percentages of one clone

NO. OF CULTURE	INITIAL NUMBER OF VIABLE CELLS PER ML	DISSOC. PERCENTAGES ON 9TH DAY
2528	280 millions	11
2529	280 millions	7
2530	280 millions	10
2531	280 millions	12
2532	280 thousands	3
2533	280 thousands	4
2534	280 thousands	4
2535	280 thousands	4

8 demonstrate inherent differences in growth rates and differences in duration of the lag phase between clones. These differences are clearly associated with the dissociation indices of the clones tested: the clone with the highest dissociation index has the fastest growth rate; clones with low dissociation indices exhibit slower growth rates and longer duration of the lag phase.

Differential Viability of Variants

Differences in viability, i.e., the ratio of the total number of cells to the number of viable cells, between smooth populations from different clones during growth in broth have so far not been thoroughly analyzed. However, significant differ-

ences in viability between smooth clones under special environmental conditions, namely, suspension in 1 per cent urea solutions, have been observed and will be reported separately. Recently, significant differences in viability during growth in broth have been found between cultures started from smooth cells and cultures started from rough cells. Both S and R had been isolated from one 10-day-old broth culture which had been started with S cells only. The results of one such test, compiled in table 9, indicate higher viability but slower growth rate in the rough culture as compared with the smooth culture.³

DISCUSSION

The foregoing experiments have revealed that inherent factors control dissociation percentages and that it is, therefore, possible to select clones with different dissociation indices. The experiments also revealed that these genetically controlled dissociation indices can be modified by changes in the environment, particularly changes which affect the growth rate and viability. Under such altered environmental conditions the absolute degree of dissociation is changed, but the relative differences between two clones, such as a high dissociating one and a low dissociating one, are retained; i.e., environmental influences which lower the dissociation index will decrease the dissociation index of a high and a low dissociating clone proportionally. Finally, a demonstration of growth rate differences between clones with different dissociation indices directly confirmed the important role of inherent growth rates in the control of dissociation indices.

When inherent differences in dissociation percentages between clones were first observed, it was thought that these might be due to a different rate of appearance of variants in different clones. However, the subsequent studies on environmental effects upon the dissociation index in selected strains suggested that the percentage of dissociation, as such, is not an inherent characteristic, but rather a secondary indicator of primary inherent differences, such as differences in growth rate and viability between clones. In order to substantiate this suggestion, it is now necessary to demonstrate that differences in growth rate and viability alone can lead to differences in dissociation percentages, even at an equal rate of appearance of variants. The data here collected permit such a demonstration, provided the dissociation phenomenon is interpreted as a process of natural change (mutation?)⁴ and selection, under the control of inherent and environmental factors. During periods of active multiplication a small percentage of variants arises continuously. The chances of these variants to establish themselves within a population (i.e., dissociation percentages) depend on their growth rates and viability within a given environment plus the degree

³ A mathematical calculation based on the data of table 9 revealed that, despite its slower growth rate, the higher viability of the R type here tested is sufficient to give the R a higher selection value than the S if they are competing within one population.

⁴ Although the work with *Brucella abortus* has not supplied any direct evidence that the actual change from one type to another is a mutational step, recent work by Humphries, Demerec, Luria and Delbrück, and others makes it highly probable that such is the case.

of population pressure existent in the population in which they arise. This population pressure, in turn, is determined by the growth rate and viability of the original members of the population.

This concept, which emphasizes the role of population dynamics and natural selection in dissociation, will now be amplified. As expressed above it necessitates the existence of population pressure for the establishment of variant types within a population. The work of Jordan and Jacobs on the growth of bacteria in liquid media and the work reported here with *Brucella abortus* have shown that, after an initial growth phase, crowding and competition between types will result. Since limits exist for the size of the viable population which can successfully maintain itself within a given amount of liquid, selection will take place. The point at which population pressure begins to act as selection pressure, producing conditions which permit the establishment of variant types with positive selection value, may vary and is determined by growth rates and viability. In figure 9 (A, B, and C) it has been attempted to illustrate the effects of differences in growth rates and viabilities upon variations in the time of the beginning of selection pressure, and it is shown how this will subsequently lead to different dissociation indices. It may be seen that if a strain A has an inherently fast growth rate, the point at which population pressure starts will be earlier in this strain than in a strain B with slow growth rate. Consequently the dissociation index (after 10 days of growth) will be higher in strain A, although the percentage of variants arising may be the same. The difference in growth rate, controlled by inherent factors in strains A and B, can also be produced by environmental factors, like pH, and different dissociation indices for one strain may thus result under different environmental conditions. Figure 9 C illustrates the possibilities for a shift in the start of selection pressure through higher viability of the original type, without change in growth rate, and the subsequent effect on dissociation indices.⁶ Finally, D in figure 9 illustrates the effect of changes in the viability or the growth rate of the arising variant upon the dissociation index. The same graph, D, would also apply to changes in dissociation indices through changes in the rate of appearance of variants (differential mutation rates); this possible *additional* control of dissociation percentages through differences in mutation rates cannot be eliminated.

It has thus been shown how inherent factors which determine growth rates and viabilities can cause the experimentally demonstrated differences in dissociation indices of different homogeneous populations, even if variants appear at a constant rate. Two additional facts may be cited in further support of this interpretation. First, the implied necessity of population pressure for the establishment of variant types within a population is substantiated by the conditions under which the appearance of variant types are most commonly observed.

⁶ Experimental evidence for this possibility has just been obtained: the growth rate of one clone used in a temperature experiment was similar at 34 C and 38 C; despite equal inocula the number of viable cells, however, differed greatly, i.e., 204 millions per ml at 38 C compared to 445 millions per ml at 34 C on the sixth day. The dissociation index at 34 C was 3 per cent, at 38 C 63 per cent.

Little dissociation has been observed when growth takes place on a solid medium. Here the chances for multiplication are physically limited, and, therefore, the population pressure is low. In liquid media, however, where dissociation is usually observed, far fewer limits exist for multiplication. Therefore, crowding and competition between types will result. This population pressure will then permit the establishment of variant types with positive selection value. Second,

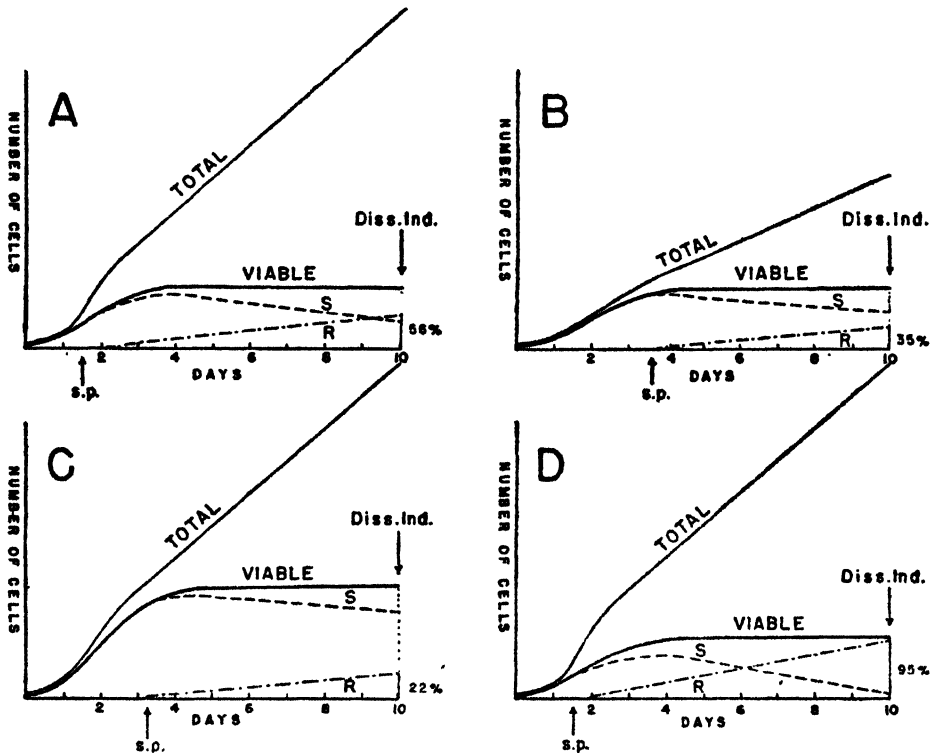


FIG. 9. DIAGRAMS ILLUSTRATING THE ESTABLISHMENT OF VARIANTS WITHIN A POPULATION THROUGH SPONTANEOUS OCCURRENCE (MUTATION) AND SUBSEQUENT SELECTION AND THE EFFECTS OF VIABILITY AND GROWTH RATE UPON THE DISSOCIATION INDEX

A. basic diagram; B. slower growth rate of original type; C. higher viability of original type; D. faster growth rate, or higher viability of variant, or higher rate of appearance (higher mutation rate). For further details see discussion. s. p. = start of selection pressure; Diss. Ind. = Dissociation Index; ---- viable S cells; - - - - viable R cells.

according to the graphs in figure 9, some time is expected to elapse before sufficient population pressure is established in cultures started with a small inoculum. Consequently, not many dissociated types are expected to be found during the early period of population growth. Experimental data substantiate this expectation, since it has been shown that dissociation can first be observed a few days after the start of a broth culture (figure 1).

It should be mentioned that a relationship between morphologic variations and growth rates in various species of bacteria has previously been described by Henrici (1928). Henrici, however, interpreted his data in terms of regular and orderly changes which a bacterium undergoes during its stages of growth (cytomorphosis), claiming that "each character reaches its maximum development in some particular phase or at some particular point of inflection of the growth curve." Without going into a detailed criticism of Henrici's "cytomorphic" interpretation, it may be stated that his data fit equally well into the concept of dissociation here advanced, especially if it is kept in mind that Henrici failed to use clones in his studies.

The interpretation of dissociation in terms of the spontaneous appearance of variants, and their subsequent establishment under the control of inherent and environmental factors governing population dynamics, is meant to apply only to the occurrence of dissociation under natural conditions of population growth. It is likely that other mechanisms may cause dissociation by a more direct action on the cell, such as X-rays (Gray and Tatum, 1944), ultraviolet rays (Haberman, 1941; Hollaender and Emmons, 1941; Braun, 1943), possibly antibodies (Dawson and Sia, 1931; Emerson, 1944), and a specific transforming substance, a form of desoxyribonucleic acid, recently isolated by Avery *et al.* (1944). (In the case of antibodies, however, the effect may not be a direct one, but rather come through the creation of a specifically selective environment due to the presence of "suppressive" antibodies.)

During the work with clones of *Brucella abortus* inherent factors other than those controlling dissociation have been recognized. One of them is the inherent control of ability to withstand toxic effects, already mentioned. Another inherent characteristic seems to be the "pattern of dissociation," i.e., whether primarily from S to R, or from S to Br. Also, some clones appear to give rise to R types which can revert to a true S type; others give rise to R types which yield the so-called S^R type (Henry, 1933). This genetic control of the "pattern of dissociation" explains why some workers observed an S^R type (Henry, 1933) and others did not (Huddleson, 1943).

It can be hoped that recognition of the role of inherent and certain environmental factors in dissociation, as well as in the control of other characteristics of bacteria, will eventually lead to an improvement of vaccines through systematic selection of clones with desired characteristics for the manufacture of vaccines. Work in this direction is now under way.

SUMMARY

With the help of single cell isolation the existence of inherent differences between clones of *Brucella abortus*, strain 19, in regard to dissociation percentages has been demonstrated under standardized environmental conditions. Clones with statistically significant differences in dissociation indices have been systematically selected and have remained stable if stored at low temperatures.

These inherent dissociation percentages can be modified by environmental changes, particularly, changes which affect growth rates and viability. Thus,

changes in pH, daily transfers, changes in temperature, differences in available nutrients, or reduced oxidation-reduction potentials alter the dissociation index of any given clone. Actual counts of bacteria revealed that environmental conditions which decrease the growth rate lower dissociation percentages. It was found, finally, that, under standard environmental conditions, clones with different dissociation indices have inherently different growth rates.

The studies on environmental effects suggested that dissociation percentages, as such, are not an inherent characteristic, but rather secondary indicators of primary inherent differences in growth rate and viability between clones. An interpretation of the experimental results is offered which demonstrates that inherent or environmentally induced differences in growth rate and in viability can cause differences in dissociation percentages, even at an equal rate of appearance of new variants. This interpretation is based on observed phenomena of population dynamics and the idea (substantiated by the work of others) that a small number of variants (mutants) arises constantly during periods of active multiplication. Counts of the number of total and viable bacteria, respectively, had shown that during population growth the total number of cells increases steadily, whereas the number of viable cells, regardless of the size of the inoculum, soon reaches a maximum which is retained as long as growth persists. At the point of population growth at which the total number of cells becomes steadily greater than the viable number of cells, population pressure starts. This population pressure can act as selection pressure and will permit the establishment of spontaneously arising variants (with positive selection value) within a population. The growth rate and the viability of the original members of a population determine the point at which population pressure starts, and the growth rate and the viability of a variant determine its chances to establish itself within a population after population pressure has started. Therefore, any changes in growth rate or in viability can produce differences in dissociation indices.

Bacterial dissociation is thus interpreted in terms of the spontaneous appearance of variants (mutants) and their subsequent establishment under the control of the inherent and environmental factors which govern population dynamics.

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STUDIES ON BACTERIAL MUTABILITY: THE TIME OF APPEARANCE OF THE MUTANT IN *ESCHERICHIA COLI*

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Students of bacterial mutability are familiar with the fact that, if a mutant occurs, it always makes its appearance with a delay of at least 3, and sometimes more than 9, days after inoculation (Parr and Robbins, 1941). It has been shown (Lewis, 1934, Yudkin, 1938; Parr and Robbins, 1941) that one reason for this delay is simply the fact that a certain time must elapse before the medium will be changed so as to become "preferential" for the eventual mutant. It is obvious that a mutant, occurring at a rate of one per million (and this is the order of magnitude of the frequency of a given bacterial mutation), would never have a chance to "show up" if the medium did not somehow favor its divisions more than the divisions of the million surrounding mother type of cells. Such a development of the preferential medium is one of the reasons for the appearance of "rough" and other mutants in old cultures. In one of his experiments (unpublished) the author was able to detect a rough mutant of *Escherichia coli* in a 30-day-old broth culture. Further investigation has revealed that, whereas the broth in the young culture (up to 10 hours) was decidedly preferential for the "smooth" mother cells, the 24-hour-old broth was already preferential for the "rough."

To eliminate this delay, a great deal of work has been done with media which are definitely preferential for the mutant from the beginning of experiment. Such are for instance the sugar-containing media used in the study of mother strains which are unable to ferment this sugar but which mutate to the sugar-fermenting variety; the latter will always divide faster on these media. Of these studies the best known are those of "*B. coli-mutabile*," especially those dealing with strains which mutate with respect to their ability to ferment lactose (see review by Parr, 1939). These strains, which originally do not ferment lactose, may develop many papillae on Endo medium in a few days. The papillae are actually colonies of cells capable of fermenting lactose. Such cells originate from a single mutant which divides much faster than the surrounding cells.

Another strain of *Escherichia coli* has been described (Parr, 1938; Parr and Simpson, 1940) which is far better for the study of bacterial mutability than the ones previously reported. This strain, almost entirely unable to utilize citrate (like typical *E. coli*), mutates to one which can fully utilize it. The mutants appear as large colonies on a very faint background of growth obtained by streaking the mother cells on citrated agar (figure 1). To avoid repetition, the reader is referred to the original literature. The author wishes only to mention



FIG. 1. FORTY-EIGHT-HOUR-OLD STREAK OF THE CITRATE-UNSTABLE STRAIN H23 ON SIMMONS (CITRATED) AGAR $\times 20$

The citrate-positive mutant appears as a large colony on a faint background growth of the citrate-negative mother cells.

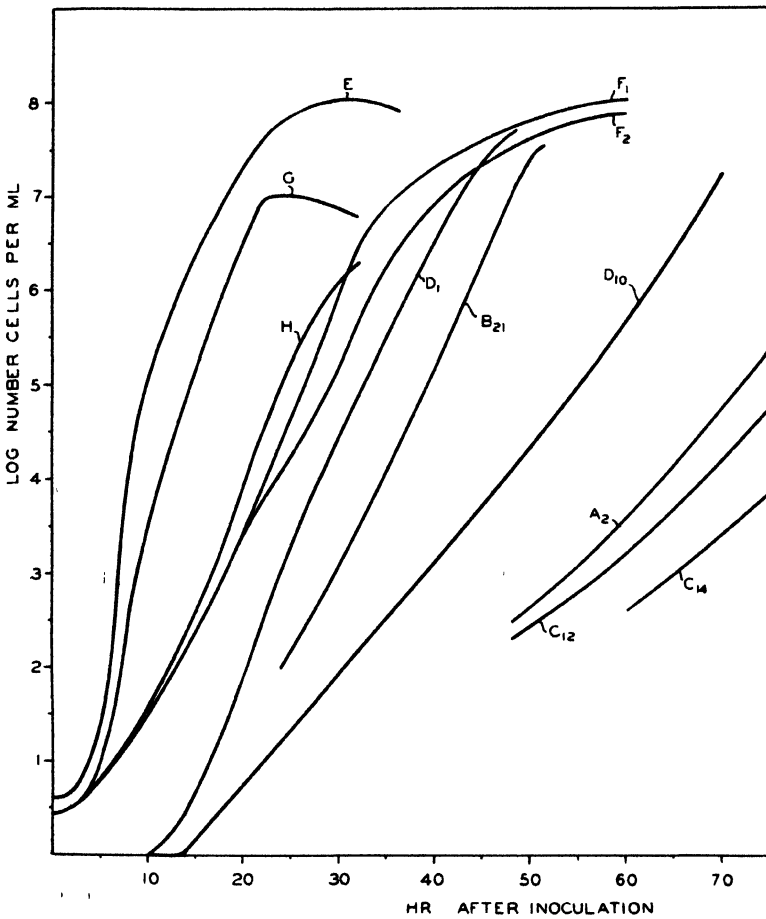


FIG. 2. GROWTH CURVES OF THE DESCENDANTS OF THE MUTANT CELLS (CURVES A TO D), AND OF THE NORMAL CITRATE-POSITIVE AND CITRATE-NEGATIVE CELLS (CURVES E TO H)

For explanation see tables 2 to 4

that the study of isolated mother cell colonies (figure 3) reveals that in the early stage the mutant cells also form papillae, as in any lactose-unstable strain. However, because of the fact that each mutant cell is able to produce up to 100 times more cells than the mother cell does, any papilla will quickly cover the entire mother colony and finally form a large colony several times the diameter of the tiny mother colony. This tremendous preference of the medium for the mutant, from the very beginning, makes this strain an invaluable tool for the study of bacterial mutability.



FIG. 3. FOUR-DAY OLD ISOLATED MOTHER CELL COLONY WITH A LARGE CITRATE-POSITIVE PAPILLA AND SEVERAL SMALL CITRATE-NEGATIVE PAPILLAE. $\times 20$

TABLE 1

Time of appearance of first citrate positive colony on streaks of citrate-unstable mother cells

TIME AFTER STREAKING	NUMBER OF STREAKS ON WHICH THE FIRST CITRATE POSITIVE COLONY APPEARED IN THE TIME INDICATED
hours	
12	0
24	0
36	24
48	33
60	24
72	16
later than 72	8

Streaks on Simmons agar, in petri dishes. Each streak made of 0.01 ml of bouillon culture of the citrate-unstable strain H23 of *Escherichia coli*; incubation at 37.5 C. Total number of streaks examined, 105.

As has been stressed by Parr and Robbins (1941), "one of the puzzling factors about the citrate mutant and about the slow-lactose-fermenting forms is . . . the delay in appearance of the mutant after the inoculation of suitable material has been made." Even though the media are undoubtedly tremendously preferential for the mutant from the very beginning, the mutant appears only after 3 days or more. The normal citrate-positive (or lactose-positive) culture if streaked on the same medium in any dilution will show growth without any delay (within 12

hours); hence the delay in the appearance of the citrate-positive (or lactose-positive) mutant is puzzling. Obviously, this phenomenon cannot be explained by the low probability of mutations because, given a large number of cases observed, some of the mutations should occur in the first 12 hours. This has never been observed, no matter how many cultures were examined (table 1; see also Parr and Simpson, 1940; Zamenhof, 1945a). In table 1 the author has summarized the results obtained from streaking the citrate-unstable strain on citrated agar. This table reveals that the number of mutants appearing is highest around 48 hours and gradually decreases before and after this time; however, before 36 hours this number sharply assumes a zero value.

The object of the present paper is to study the reasons for this delay in the appearance of the mutants.

EXPERIMENTAL

The work was done entirely on the H23 citrate-unstable strain of *E. coli* studied by Parr and Simpson (1940). In order to obtain quantitative results, citrated broth (Koser's) rather than citrated agar was used as the medium in which the mutants are supposed to appear within a certain time after inoculation.

As inoculant in each series (tables 2 and 3) a different bouillon culture of the H23 strain was used. Each of these cultures was obtained from a single, isolated, 12-hour-old colony on citrated agar (Simmons'). The cultures were tested separately on Simmons' agar and in Koser's broth and proved to be of the pure "citrate-unstable" strain, entirely free from citrate-positive cells. Further check was made to ascertain that the culture did not contain any appreciable number of the stable citrate-negative cells (i.e., cells which never mutate to

TABLE 2

Growth of the descendants of mutant cells

(Growth in 10 ml of Koser's broth with 0.1 per cent bouillon; strain H23; incubated at 37.5°C.)

C. Method used: streaking 0.01-ml portions of culture. The figures correspond to the numbers of cells found in each 0.01-ml portion)

HR AFTER INOC	SERIES A, TUBE NO.											
	1	2	3	4	5	6	7	8	9	10	11	12*
12	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
36	2	0	0	0	0	0	0	0	0	0	0	0
48	60	3	0	2	0	10	90	26	20	9	0	19
60	600	38	11	3	0	140	160	37			28	34
72	T	850	750	600	4	T			300		160	1,000
84		T	T	T								T
96					1,400				T	2,000	T	
108					T					T		

Letter T denotes the appearance of turbidity in broth.

* Tubes 13 to 16 showed no cells.

HR AFTER INOC	SERIES B, TUBE NO																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0
36	1,000	800	2,000	220	1,000	600	160	4	320	1,000	170	170	1,200	0	0	1,500	127	1,200
48	T	T	T	T	T	T	T	303	T	T	T	T	T	16	500	T	T	T
60								T							T			
72																		
84														T				

HR AFTER INOC	SERIES B, TUBE NO. (CONTINUED)																	
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35*	
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
24	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
36	600	620	200	1,200	1,300	800	0	0	0	0	0	17	120	0	0	0	0	
48	T	T	T	T	T	T	230	0	0	0	55	1,200	1,500	0	0	0	0	
60							T	150	0	118	T	T	T	13	117	200	300	
72									0							T	T	
84							T	1,200	T				1,500	T				
96								T						T				

* Tubes 36 and 37 showed no cells

HR AFTER INOC	SERIES C, TUBE NO										
	1	2	3	4	5	6	7	8	9	10	11
12	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	1	0	0	0	0	0	0
36	0	0	0	0	14	18	0	500	0	800	295
48	6	35	4	126		57	10	T	57	T	T
60	204	1,000	1,000	1,200	T	157	2,000		2,200		
72	T	T	T	T		T	T		T		

HR AFTER INOC	SERIES C, TUBE NO. (CONTINUED)									
	12	13	14	15	16	17	18	19	20	21*
12	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0
48	2	0	0	5	0	0	0	0	0	0
60	17	39	4	99	0	0	0	0	22	0
72	300	2,000	45	1,500	0	0	12	0	382	0
84					0	0		0		0
96	T	T		T	150	0		21	T	7
108			T			0				
120					T	54	1,000	500		
132							T			T
144						T		T		

* Tubes 22 to 27 showed no cells.

citrate-positive and which might have originated from the original strain by mutation). The check consisted in streaking the culture in a dilution of 10^{-7} on several Simmons' agar plates; from the 140 well-isolated colonies obtained in this way, 131 developed citrate-positive papillae within 40 to 96 hours. This proves that for all practical purposes the original culture did not contain stable citrate-negative cells. This does not mean, however, that such a mutation is impossible (see Parr and Simpson, 1940), but merely that it has negligible influence for the quantitative approach presented in this paper.

It is interesting to note the behavior of a stable citrate-negative strain under the same conditions: 50 well-isolated colonies remained perfectly smooth and never showed any papillae, although they would have shown abundant papillae

TABLE 3

Growth of the descendants of mutant cells

(Growth in 10 ml of Koser's broth with 0.1 per cent bouillon, strain H23; incubated at 37.5 C. The figures correspond to the numbers of cells in 1 ml)

Series D

HR AFTER INOC- ULA- TION	MF- THOD	TUBE NO									
		1	2	3	4	5	6	7	8	9	10*
10	I	1	0	0	1	1	0	1	0	0	1
14		3	37	0	2	2	0	4	1	1	1
22		340	120	10	414	184	59	16	28	2	13
26	II	3,100	165	206	700	1,000	110				314
36		5×10^5	1.6×10^4	2×10^4	1.6×10^4	2.8×10^5	9.6×10^4	700		400	154
48		5×10^7	2×10^7	5×10^7	10^8	10^8	5×10^7			3×10^4	1.2×10^4
58					10^8			5×10^7	10^8	10^7	3.3×10^5
70											2×10^6

Method I: Pouring 1-ml portions of culture

Method II: Streaking 0.01-ml portions of culture (computed for 1 ml)

* Tubes 11 to 13 showed no cells

if the culture had been streaked on Endo medium. This proves the independence of these two kinds of mutations (citrate and lactose).

In series A, 16 tubes, each containing 10 ml of Koser's broth, were each inoculated with 0.01 ml of the inoculant and then incubated at 37.5 C.

In series B, 0.40 ml of the inoculant were added to a flask containing 400 ml of Koser's broth. The culture was incubated for 12 hours and then divided into 40 portions of 10 ml each, which were incubated again. This method gives a rather uniform bacterial content in each tube.

In series C, 0.40 ml of fresh bouillon were added to a flask containing 400 ml of Koser's broth. The flask was inoculated with 0.03 ml of a 5.4×10^{-6} dilution

of the inoculant culture; these 0.03 ml were found to contain 27 cells. After 12 hours the contents of the flask were divided into 40 portions, as in series B.

With one method, the early presence of mutants and their multiplication in experimental Koser's broth was tested by streaking, every 12 hours, 0.01 ml of this broth on citrated agar (Simmons'). The appearance of turbidity in each tube was also recorded. This turbidity means, of course, the abundant multiplication (3×10^7 to 2×10^8 cells per ml) of citrate-positive cells, because citrate-negative cells (of the H23 strain) are able to multiply only up to about 2×10^6 cells per ml, which is not enough to give turbidity.

Early in this work it was found that, if pure Koser's broth is used and the inoculant does not contain bouillon, the quantity of citrate-negative cells is not higher than 2×10^6 per ml, and the delay in the appearance of turbidity is usually longer than 5 days. If bacterial "mutations" are at all comparable to the mutations in higher organisms, then the probability that a mutation will occur must somehow increase with the increasing number of cells or the number of bacterial divisions (Zamenhof, 1945a). Obviously, the larger the number of cultures, the higher is the probability that in one of them the mutant will occur within a given time. The author has found that the same increase in probability can be obtained by using volumes of broth larger than 10 ml.

A more convenient method, used throughout the later work, was to add to Koser's broth 0.1 per cent of bouillon; this was added as fresh bouillon when the inoculant was diluted, or as old bouillon when undiluted inoculant was used (i.e., when the inoculant was 0.01 ml of the old broth). In both cases this addition allows one to obtain as many as 10^7 cells per ml. It has also been found that a still better medium is obtained by heating the turbid citrate-positive culture in Koser's broth to 60 C'.

It should be noted that the addition of bouillon in large quantities tends to destroy the preference of the medium for the citrate-positive cells because it improves the conditions for the citrate-negative cells. However, the experiments have shown that, in the proportion of 0.1 per cent, the bouillon does not impair preference and gives a very convenient method for obtaining earlier appearance of the mutant. As will be seen below, it also helped to create conditions in which the first mutant cells can be detected without delay.

The method previously described of streaking 0.01 ml of culture is not convenient for detecting the first citrate-positive cells in the culture. A method, described below (series D), is better adapted for this purpose and has been used for the data in the first part of table 3.

The 10-ml cultures are incubated as usual. Every few hours 1-ml portions of each culture are taken to prepare poured plates (with 10 ml of liquid Simmons' agar). If any citrate-positive cell is present at this time, it will appear within 12 to 24 hours as a large, deep (or surface) colony. This method, although not testing the entire 10 ml, gives a rather close estimation of the time of appearance of the first few citrate-positive cells.

For comparison with the previous method, and also for evaluation of further

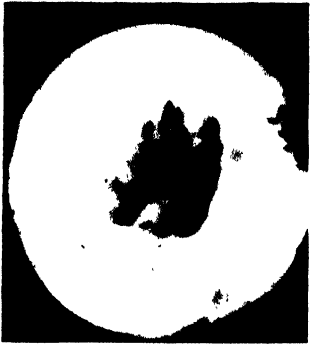


FIG. 4. ONE-WEEK-OLD CITRATE POSITIVE PAPILLA WHICH HAS COMPLETELY COVERED THE FAINT MOTHER COLONY AND HAS PRODUCED NUMEROUS SECONDARY CITRATE POSITIVE PAPILLAE. $\times 20$

TABLE 4

Growth of normal citrate-positive and citrate-negative strains of Escherichia coli
(The figures correspond to the number of cells in 1 ml)

HR AFTER INCUBATION	SERIES E	SERIES F ₁	SERIES F ₂	SERIES G	SERIES H
6	100				
9	5.85×10^4			1,400	
13	7.5×10^5			3.2×10^4	100
21		5×10^3	4.1×10^3		
22	4.27×10^7	1.06×10^4	6.4×10^3	9.5×10^6	1.13×10^5
	T				
28	9.3×10^7			8.5×10^6	7.2×10^5
30		8.34×10^5	1.47×10^5		
32				6.13×10^6	2×10^6
				C	C
34		7.28×10^6	1.41×10^6		
36	8×10^7	1.135×10^7	2.93×10^6		
45		2.81×10^7	2.3×10^7		
		T	T		
60		1.05×10^8	7.5×10^7		

Series E: Citrate-positive cells inoculated into fresh Koser's broth with 0.1 per cent bouillon.

Series F₁: Citrate-positive cells inoculated into a 13-hour-old culture of a citrate-negative strain in Koser's broth with 0.1 per cent bouillon.

Series F₂: Same as F₁ but inoculated into a 22-hour-old culture of a citrate-negative strain.

Series G: Citrate-negative cells inoculated into fresh Koser's broth with 0.1 per cent bouillon.

Series H: Citrate-negative cells inoculated into fresh Koser's broth without bouillon.

Letter T under numbers denotes appearance of turbidity in broth; C means clear broth.

divisions, the 0.01-ml portions of the cultures after 26 hours were streaked as previously described. The results are entered in the second part of table 3.

For comparison with the behavior of normal citrate-positive and citrate-negative strains, growth rates (table 4) were also determined (the data being averages of at least three tubes of each series). Series E shows the growth of the normal (pure) citrate-positive strain in 10 ml of Koser's broth to which 0.01 ml of fresh bouillon was added. The inoculant was 0.03 ml of a 5.4×10^{-6} dilution of a 24-hour-old Koser broth culture of this strain; these 0.03 ml contained a total of 40 cells. Series F₁ and F₂ show the growth of the same strain inoculated into 10 ml of 13-hour-old (series F₁) and 22-hour-old (series F₂) Koser broth cultures of a citrate-negative strain. The broth also contained 0.01 ml of bouillon. Series G shows the growth of a citrate-negative (stable) strain in 10 ml of Koser's broth with 0.01 ml of fresh bouillon. Series H is the same as series G, but without 0.01 ml of bouillon.

The results are summarized in tables 2 to 4 and represented graphically in figure 2. It should be mentioned that the curves of the A to D series represent the growth of the descendants of the citrate-positive mutant. Only a few (typical) curves of these series are shown. Study of the tables and diagrams seems to indicate the following:

(1) Given a sufficient number of mother cells, there may be absolutely no delay in the occurrence of the first mutant. Table 3 shows that the mutant may occur earlier than the tenth hour, that is, even before the number of mother cells reaches the highest level.

(2) The reason for the delay in the detection of mutants, observed by the previous investigators, is not to be seen only in the small number of mother cells (i.e., low probability). As stated before, even if the number of mother cells in each tube (or on each agar streak) is small, yet, given a sufficient number of cases, the mutant in some of them would appear within 12 hours. This is clearly shown by the steep growth curve E of the normal citrate-positive strain in fresh Koser's broth. The reason for the delay in the detection of mutants must therefore be sought somewhere else. The reason appears to be revealed by the growth curves of the descendants of the first mutant cell. These curves, and especially D₁₀ to C₁₄ in figure 2, indicate that the multiplication of the descendants of the first mutant cells is very slow as compared with that of the normal citrate-positive cells under the same conditions (curve E). In the case of curve D₁₀ the growth has resulted in the turbidity of the tube only after 70 hours, and in the case of curve C₁₄ after 108 hours. The citrate-positive cells from such turbid tubes, if tested again in fresh broth, behave like "normal" citrate-positive cells and give turbidity within 18 hours.

DISCUSSION

In a previous communication (Zamenhof, 1945a) the author was unable to explain the delay in the detection of the mutants and he deduced that the reason may be in the old age of cells which may divide "improperly," forming mutants. Such a behavior would make bacterial mutations somewhat different from mutations in higher organisms in which the process of mutation appears to be a regular (although rare) phenomenon in the normal, healthy, young and old cells (review in Dobzhansky, 1941; Zamenhof, 1944). The present experiments

seem to indicate clearly that bacterial mutability is also a regular (although very rare) phenomenon occurring in normal, healthy, young and old cells. In that respect bacterial mutations (at least in the strains studied) would be similar to mutations in higher organisms. There is, of course, no proof that all the bacterial mutations behave this way; however, in another strain of *Escherichia coli* (Zamenhof, 1945b) there were found mutations (lactose fermentation and chain formation) which also seem to occur without any delay.

The second result, that the delay in the detection of mutants is due merely to the slow multiplication of the newly formed mutants and their early descendants, is explained as follows: As mentioned previously, the mutant or its early descendants, when streaked on fresh Simmons' agar or inoculated into fresh Koser broth, multiply as fast as normal citrate-positive cells. In contrast to this, the delay in a culture seems to indicate that the mutant and its early descendants are affected by the products of metabolism of the mother cells.

It has been shown that the first mutant occurs at the moment when the number of mother cells is high enough to make a mutation probable (table 3). At this age the products of metabolism have already reached a certain concentration and the cells may be more or less poisoned by these products. It is probable that despite the genetical change which improves the citrate utilization, the cell does not recover quickly in an old broth, especially because the concentration of the products of metabolism is still increasing. Inspection of figure 2 reveals the fact that the later the mutant occurs the less steep is its growth curve. This also seems to support the theory that the delay is caused by cell poisoning.

In the experiments and computations it has been tacitly assumed that the citrate-positive cells found in any tube are all descendants of only one original mutant. Actually, the appearance of a second mutant is also possible. Because of the low mutability rate, however, the chances are that in most cases this mutant will appear so late that its descendants will be insignificant as compared with the already numerous descendants of the first mutant.

SUMMARY

This study deals with the problem of the well-known but hitherto unexplained delay in the occurrence of bacterial mutants. To eliminate part of this delay, namely, the delay in the production of a medium preferential for the mutant, the work was done on citrate-unstable strains of *Escherichia coli*, using citrated media, which are always strongly preferential for the citrate-fermenting mutant. To make possible quantitative study, the experiments were performed on a large number of Koser broth cultures. The following facts were established:

- (1) Given an adequate number of mother cells there is no delay in the occurrence of the first mutant cell. The mutant may occur as early as within 10 hours after inoculation, that is, among young and normal cells. In that respect, these bacterial mutations may resemble mutations in higher organisms.

- (2) The reason for the delay in the detection of this mutant cell has been found in the slow multiplication of the mutant and its early descendants in the

mother culture. This slowness may be caused by poisoning by an inhibitory factor produced by the mother cells. If the mutant cells are transferred into a fresh medium, they multiply rapidly after a short lag period. Similar but less accentuated delay due to poisoning has been found in the normal citrate-positive cells if they are inoculated into 12-hour-old, or older, citrate-negative cultures.

(3) One pure mother strain may give rise to entire gamuts of mutants differing in the degree of citrate utilization, and probably also in the degree of their resistance to inhibitory factors.

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BACILLIN, A NEW ANTIBIOTIC SUBSTANCE FROM A SOIL ISOLATE OF BACILLUS SUBTILIS

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Aerobic sporeforming bacteria have long been known to produce antibiotic substances. Waksman (1945) gives a thorough coverage of the literature on the subject. However, detailed chemical and microbiological studies have been made of only a few of the substances produced by this group of organisms. Most notable have been those of tyrothricin formation by *Bacillus brevis* (Hoogerheide, 1944; Hotchkiss, 1944) and of the closely related gramicidin S of Russian investigators (Belozarsky and Passhina, 1944). Other substances studied were subtilin produced by *Bacillus subtilis* (Jansen and Hirschmann, 1944), the active material produced by *Bacillus mycoides* (Kimmelstiel, 1924), that by *Bacillus simplex* (Katznelson, 1942), and the material elaborated by *Bacillus mesentericus* (Christensen and Davies, 1940). After the work reported in this paper was completed, the isolation of the antibiotic bacitracin from a *B. subtilis* type sporeformer was described (Johnson *et al.*, 1945).

Isolation of the antagonistic soil bacillus. A particularly active isolate of *B. subtilis* was obtained from soil during a study of organisms antagonistic to *Mycobacterium tuberculosis*.

The soil was plated out in peptone glucose agar containing a suspension of live cells of *M. tuberculosis* 607. The plates were placed at 30 C to allow development of the soil organisms, the mycobacteria meanwhile not developing at this unfavorable temperature. After 4 days the plates were placed at 37 C for 3 days to permit growth of the seed *M. tuberculosis* cells. All soil colonies that were surrounded by a clear zone, indicative of inhibition of *M. tuberculosis* by the preformed antibiotics, were isolated in pure culture for further study. This pregrowth technique allows detection of the largest possible number of the antagonists in the soil population. This technique is considerably more successful than the usual methods for isolating antagonists.

Of several actinomycetes, fungi, and bacteria exhibiting antimycobacterial action, the aerobic sporeforming bacillus was selected for further study because it formed an exceptionally large zone of inhibition.

Description and identification of the antagonist. According to *Bergey's Manual*, the biochemical characteristics of this organism place it in the *B. subtilis* group and it was later identified as *B. subtilis*.² No gas was produced during growth in peptone broth containing 1 per cent of the various common diagnostic sugars. The pH values after 4 days' incubation indicated that acid was produced from

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² We are grateful to Dr. N. R. Smith of the U. S. Department of Agriculture for his confirmation of this diagnosis.

sucrose, fructose, glucose, inulin, and probably from xylose and arabinose. Acid was formed in milk, but after 4 days there was no curd formation or casein digestion.

Bacillin: differentiation from other antibiotics produced by aerobic sporeforming bacteria. In the early stages of the work with the bacillus, antibiotic activity could be demonstrated only when the organism was grown on solid agar media. Preliminary observations were made by streaking the bacillus across the middle of an agar plate, and then, after 48 hours' incubation of the plate at 30 C, cross streaking with test bacteria. The size of the inhibition zone after 2 days' further incubation served as a rough relative measurement of the amount of antagonistic material produced and of the sensitivity of the test organism. Table 1 reports a comparison by this method on several media of the antagonistic properties of this *B. subtilis* strain and *B. brevis*, the tyrothricin-producing organism.

TABLE 1
Antagonistic properties of Bacillus subtilis and Bacillus brevis

MEDIUM	B. SUBTILIS		B. BREVIS	
	Inhibition against			
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
	mm	mm	mm	mm
Yeast extract agar.....	3	10	0	2
Yeast extract glucose agar... ..	15	>15	3	3
Asparagine glucose agar.	27			
Nutrient agar.....	3	>15	0	3
Brain heart agar.....	0	>10	2	2
Peptone, glucose.....	13	>13	0	1
Peptone, glucose + m/15 PO ₄ , pH 7.0	12	14	0	3

Clear differences between the two organisms are apparent. *B. brevis*, ordinarily a potent strain, showed only slight antagonistic activity under the conditions employed, probably because tyrothricin is retained mostly in the cells and also possibly because tyrothricin, composed of large polypeptides, does not diffuse rapidly. The *B. subtilis*, on the other hand, showed two types of activity, both indicated by large inhibition zones. On organic media containing no carbohydrate, activity was limited largely to *Staphylococcus aureus*, but if utilizable carbohydrate was present, *E. coli* was also inhibited. This was not true of *B. brevis* tested under the same conditions. The data suggest the formation by the soil bacillus of two distinct antibiotic substances, depending upon the culture medium used. The anti-coli substance was named "bacillin."

Comparison of the antibacterial spectra of bacillin and of the active fraction²

² For simplicity this material is referred to as "simplexin." In this work a simplexin concentrate was prepared by norite adsorption of a corn steep liquor culture and elution with methanol. The methanol was evaporated *in vacuo* and the residue taken up in water.

from a *B. simplex* culture originally isolated by Cordon and Haensler (1939) demonstrates that these two materials are different (part A, table 2).

Bacillin was also proved to be distinct from subtilin. Active liquid asparagus juice cultures of the subtilin-producing strain of *B. subtilis*⁴ W. R. R. L. 370 were prepared for assay exactly according to the procedure of Humfeld and Feustel (1943). Marked differences were evident in the antibacterial activities of the two substances against various bacteria, as part B in table 2 demonstrates. Similar differences for other test organisms leave no doubt that the antibacterial spectra of bacillin and subtilin are quite distinct. Other differences were apparent. Bacillin activity occurs almost exclusively in the culture liquid,

TABLE 2

Comparative antibacterial spectra of antibiotics from 3 different aerobic sporeformers*

A. Bacillin and simplexin

ORGANISM	RATIOS OF AMOUNT OF ANTIBIOTIC MATERIAL REQUIRED FOR INHIBITION WITH <i>E. COLI</i> TAKEN AS THE UNIT	
	Bacillin	Simplexin
<i>E. coli</i>	1.0	1.0
<i>Eberthella typhosa</i>	0.5	1.0
<i>Salmonella paratyphi</i>	0.09	>32
<i>Pasteurella sp.</i>	0.37	0.13
<i>Diplococcus pneumoniae</i> type III.....	1.0	0.13
<i>Staphylococcus aureus</i> H.....	0.37	>32

B. Bacillin and subtilin

ORGANISM	RATIOS OF AMOUNT OF ANTIBIOTIC MATERIAL REQUIRED FOR INHIBITION WITH <i>S. AUREUS</i> TAKEN AS THE UNIT	
	Bacillin	Subtilin
<i>S. aureus</i> H.....	1.0	1.0
<i>S. paratyphi</i>	0.25	10.0
<i>Micrococcus conglomeratus</i>	2.0	1.0
<i>E. typhosa</i>	1.4	>10.0
<i>E. coli</i>	2.7	>10.0

* Streak method on nutrient agar.

very little remaining in the cells, whereas almost all the subtilin activity remains in the cells and must be extracted therefrom. The subtilin extraction procedure of Jansen and Hirschmann (1944) is not at all successful with bacillin.

The ability of bacitracin to protect animals against bacterial infections and its activity in organic media differentiate it from bacillin (see below). The antibiotic substances from *B. mesentericus* and *B. mycoides*, mentioned earlier in this paper, are insufficiently described to permit a comparison with bacillin.

Production of bacillin. The presence of a utilizable carbohydrate enhances production of bacillin by *B. subtilis*. This was demonstrated by experiments

⁴ We are grateful to Dr. H. Humfeld for supplying this organism.

in which aqueous extracts of agar cultures of the bacillus were tested in various dilutions for their ability to inhibit *E. coli* by the streak agar plate method.⁵ In such experiments the nutrient agar used for testing usually contained a small amount of tyrothricin (50 μ g per ml), which was very effective in preventing development of spores of *B. subtilis* in the medium, thus eliminating the need for Seitz filtration. Considerably higher concentrations of tyrothricin had no effect on the growth of *E. coli*. Table 3 shows that glucose, fructose, and sucrose were most effective in promoting the anti-coli activity in *B. subtilis*. Good activity could also be obtained in sugar media when the peptone or asparagine was replaced by histidine, α -alanine, or proline. Small activity was obtained on media containing ammonium salts as the only nitrogen source.

Replacement of the agar by gelatin, or reduction in the concentration of agar, led to reduced activity. Washing the agar twice with distilled water removed some of the factor presumably present which promotes growth, although some growth with lower activity was generally obtained on washed agar medium. Neither small amounts of yeast extract nor vitamin-free casein had the activity-promoting properties of agar, although these substances were capable of sup-

TABLE 3

Effect of carbohydrate on the production of anti-coli substance (bacillin) by B. subtilis

MEDIUM	DILUTION* INHIBITING <i>E. COLI</i>
Glucose peptone agar.....	8
Glucose asparagine agar.....	10
Sucrose asparagine agar.....	12
Fructose asparagine agar.....	8
Galactose asparagine agar.....	<4
Glycerol asparagine agar.....	5
Asparagine agar.....	<4

* Test medium = nutrient agar containing tyrothricin (50 μ g per ml).

porting good growth. It became evident that the unwashed agar was the only ingredient leading regularly to high activity. It appeared that an impurity was capable of stimulating bacillin production, but was not vitally essential for growth.

Tap water was found capable of replacing agar and permitted for the first time good growth and bacillin formation in broth cultures. While a study of the heavy metal fraction of tap water was in progress in this laboratory, a report by Jansen and Hirschmann (1944) appeared showing that manganese promoted subtilin formation by their strain of *B. subtilis*. Manganese salts also proved to be the factor in agar and in tap water responsible for the promotion of bacillin formation by our strain of *B. subtilis* (table 4).

Purification of bacillin. As mentioned previously, bacillin is found mostly in the cell-free filtrate of *B. subtilis* cultures, being in this respect quite different

⁵ The more accurate Oxford cup assay procedure has now been developed, employing glucose asparagine agar and *E. coli* as the test organism.

from tyrothricin and subtilin, which are found predominately in the cells of the bacteria producing them. With the demonstration that manganese was the mineral essential for the production of activity in liquid cultures, it became possible to prepare sufficient culture material to carry through preliminary purification steps. All attempts toward purification were directed toward bacillin, the antibiotic fraction active against *E. coli* as the test organism.

Preliminary stability studies on solutions containing crude bacillin showed the substance to be stable at pH 2.0 for $\frac{1}{2}$ hr at 30 C, and at 100 C for $\frac{1}{2}$ hr at pH 6.5. No loss of activity resulted from the addition of 4 volumes of ethanol. Ether extraction at pH 2.0 or 6.5 did not remove a significant amount of activity. Treatment with 2 per cent norite removed all the activity from broth.

The active fraction could not be eluted from norite with salt solutions, but was eluted with ethanol and methanol provided these solvents contained at least 5 per cent water. Acid alcohol did not improve the elution. Bacillin produced in glucose asparagine medium or glucose alanine medium always yielded a norite eluate considerably more potent than that resulting from bacillin

TABLE 4
*Effect of trace elements on bacillin production**

SALT ADDED	DILUTION INHIBITING <i>E. COLI</i> IN TYROTHRICIN AGAR
None.....	4-6
20 ppm $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	6-8
20 ppm $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	48
20 ppm $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	4-6
Combination of above	24

* Glucose, alanine, salts broth.

produced in glucose peptone medium; hence a synthetic medium (glucose and alanine) containing MnSO_4 was used in all subsequent work.

One preparation obtained by elution from norite with 90 per cent ethanol and concentration to dryness *in vacuo*, and then solution in water, assayed 110 dilution units per mg in nutrient tyrothricin agar. It contained reducing substances equivalent to 32 per cent calculated as glucose, and amino acids equivalent to 36 per cent calculated as α -alanine and determined by formol titration. Probably these represent unchanged medium constituents carried along during the purification procedure. The most potent preparation so far obtained inhibited *E. coli* in glucose asparagine medium at a concentration of 0.67 μg per ml, or 1 part in 1.3 million.

Further attempts at purification of the bacillin by solvent extraction, by precipitation with mercury, silver, or lead salts, or by phosphotungstic acid, 2,4-dinitrophenylhydrazine, and acid precipitation were unsuccessful. There was a fractionation of activity, but this was to no advantage on a potency basis. Hydrogen sulfide completely abolished the activity of bacillin.

Antibacterial spectrum of bacillin in various media. A liquid concentrate

prepared as was described above and assaying 50 units per mg solids in nutrient agar containing tyrothricin was sterilized by passage through a glass bacterial filter, and its antibacterial spectrum was determined in various media as listed in table 5. The range of antibacterial activity against the different organisms tested was rather uniform on any one medium, considering the extreme variations generally encountered with antibiotics.

Gram-negative and gram-positive bacteria were affected by fairly similar concentrations. A particularly noticeable observation was that there appeared to be a strong neutralizing effect by the more complex media upon the inhibitory action of bacillin. For example, *E. typhosa*, inhibited by a 1,000 dilution in

TABLE 5
*Antibacterial spectrum of bacillin in different agar media**

TEST ORGANISM	GLUCOSE ASPARAGINE	NUTRIENT + 0.01% TYROTHRICIN†	NUTRIENT	NUTRIENT + 0.5% BLOOD	BRAIN HEART
<i>E. coli</i>	640-960	240-320	120-160	20	<10
<i>E. coli</i> W†.....	640-960	160	80-120	20	<10
<i>E. typhosa</i> ‡.....	640-960	960	240	40	20
<i>E. typhosa</i>	960-1,280	480-640	240-320	20	<10
<i>S. paratyphi</i>			1,280	40-80	10-20
<i>S. schottmuelleri</i>	960-1,280	120	80	20	<10
<i>S. schottmuelleri</i> ‡.....	640-960	240	80	20	<10
<i>Pasteurella</i> sp.....	960-1,280	640-960	320	<10	<10
<i>M. tuberculosis</i>	640-960				
<i>D. pneumoniae</i> type I.....			480-640	10	20
<i>D. pneumoniae</i> type III.....			120-160	10-20	10
<i>S. aureus</i> H.....			320-480	20-40	20
<i>S. albus</i>			320-480	10	20
<i>Staphylococcus</i> (Smith)‡.....			320-480	80	40
<i>Streptococcus</i> ‡.....				960-1,280	20-40
<i>M. conglomeratus</i>			160-240	10	10-20

* Figures represent dilutions. Where 2 figures are given, inhibition, though pronounced at the higher dilution, was complete only in the lower one.

† This concentration of tyrothricin just prevents development of spores of *Bacillus subtilis* present in unsterilized bacillin preparations.

‡ Cultures kept virulent through frequent animal passage.

synthetic glucose asparagine medium, was not inhibited in brain heart agar by the same bacillin solution diluted only 1:10. Brain heart agar, nutrient agar containing 0.5 per cent whole rabbit blood, and nutrient agar alone all behaved similarly, and in that order of effectiveness. The neutralizing action was general for all bacteria so far tested. This effect is the subject of a detailed study presented in the next paper.

*Animal experiment.*⁶ Bacillin concentrates showed only moderate toxicity for mice. Twenty mg injected intraperitoneally was the highest tolerated dose. However, this amount of bacillin was totally ineffective in preventing the deaths

⁶ We wish to thank Mr. Otto Graessle for these animal tests.

of mice infected with virulent strains of *S. aureus* and *Diplococcus pneumoniae*. Concentrates of simplexin also showed no protection at the highest tolerated dose (20 mg). Presumably the inactivating effect of blood previously described accounts for the failure of bacillin to protect the animals against the infections.

SUMMARY

A new antibiotic, bacillin, has been obtained from a soil isolate of *Bacillus subtilis*. Bacillin is highly active against gram-negative and gram-positive bacteria in certain media. Its differentiation from known antibiotics from similar bacteria, the conditions relating to its production in solid and in liquid media, the purification procedure for obtaining highly potent concentrates, and some chemical properties are described. Crude bacillin concentrates are moderately toxic for mice and completely ineffective in protecting the animals from virulent bacterial infections. The presence in the medium of blood and other complex natural materials reduces or abolishes the antibacterial properties of bacillin.

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ANTIBACILLIN, A NATURALLY OCCURRING INHIBITOR OF BACILLIN

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The observation that the antibacterial activity of bacillin is reduced or abolished in certain complex organic media (see preceding paper) led to the suspicion that one or more specific chemical substances might be responsible for this neutralizing effect, and, accordingly, study of this was undertaken together with an attempt to purify the active substance(s). Besides occurring in the complex constituents of the usual organic media, the inhibitor is present in blood and possibly in other tissues and body fluids of animals. This probably accounts for the failure of bacillin to protect mice against virulent infections with susceptible bacteria. Active concentrates of the bacillin inhibitor have been prepared, and the active fraction has been named "antibacillin."

Occurrence in natural materials. The existence of the bacillin-neutralizing factor in many naturally occurring complex organic materials could easily be demonstrated by the addition singly of small amounts of the various materials to a basal assay medium containing graded doses of bacillin, inoculation with *Escherichia coli*, and incubation overnight at 37 C. The neutralizing effect of the substance on bacillin is indicated by the considerably larger amounts of bacillin required for inhibition in the presence of the organic supplement than in a supplement-free control.

Table 1 shows that small amounts of certain materials are extremely successful in reducing the effectiveness of bacillin as an antibacterial agent. Most notable in this respect are brain heart infusion, N-Z-case, tryptone, whole rabbit blood, and rabbit serum. It took 128 times as much bacillin to inhibit *E. coli* in the presence of 10 per cent whole rabbit blood as in its absence. This strong neutralizing power of blood doubtless explains why bacillin is ineffective as a therapeutic agent in the animal body. Peptone was definitely active but to a lesser degree, whereas beef extract and liver extract appeared to have some activity. In view of the high activity of rabbit blood, the complete lack of activity in Difco dried blood is surprising, but the activity may have been destroyed during processing.

Cephalin, known to counteract the antibacterial action of tyrothricin (Hotchkiss, 1944), was without effect on bacillin, further proof of the dissimilarity of the two antibiotics. As will be shown later, higher concentrations of inorganic salts are able to depress, nonspecifically, bacillin activity, but in this experiment NaCl equivalent to 10 per cent of the organic materials added was without effect.

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Thus, all of the organic extracts and digests tested and used commonly in bacteriological media showed clear-cut antibacillin activity with the exception of beef blood and beef extract.

Liberation by acid hydrolysis. Since all of the active materials have undergone a certain amount of hydrolysis during their preparation, a more extensive survey was made of naturally occurring complex materials, comparing the untreated material with a corresponding set hydrolyzed with 2N H_2SO_4 for 1 hour at 120 C and neutralized with $\text{Ba}(\text{OH})_2$. The BaSO_4 was removed by filtration. Synthetic glucose asparagine agar was used as a base in this experiment, with *E. coli* as the test organism.

Table 2 shows that hydrolysis led to a decided increase in the antibacillin

TABLE 1
*Inhibitory effect of complex organic materials on bacillin activity**

SUBSTANCE ADDED	AMOUNT PER ML AGAR	DILUTION OF BACILLIN CONCENTRATE INHIBITING <i>E. COLI</i> †
None.....		24,000-48,000‡
Brain heart infusion, Difco..	10 mg	375-750
N-Z-case\$, Sheffield Farms ..	10 mg	375-750
Tryptone, Difco.....	10 mg	750-1,500
Peptone, Difco	10 mg	3,000-6,000
Yeast extract, Difco	10 mg	6,000-12,000
Beef extract, Difco.....	10 mg	12,000-24,000
Liver extract, Wilson	10 mg	12,000-24,000
Beef blood, Difco	10 mg	24,000-48,000
Fresh whole rabbit blood	0.1 ml	188-375
Fresh rabbit serum	0.1 ml	1,500-3,000
Cephalin.....	1.0 mg	24,000-48,000
NaCl.....	0.1 mg	24,000-48,000
NaCl.....	1.0 mg	24,000-48,000

* Basal test medium = 1 per cent glucose, 0.5 per cent asparagine, 0.1 per cent phosphate buffer pH 7.0, 0.025 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001 per cent $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 per cent agar.

† Streak method on agar plates; overnight incubation at 37 C.

‡ Throughout this paper, whenever 2 dilution figures are given, inhibition, though pronounced at the higher dilution, was complete only in the lower one.

§ A tryptic digest of casein.

content of most of the materials tested. Milk, however, was extremely active in the unhydrolyzed state, and its activity was not increased appreciably through hydrolysis. Special note is made of the fact that the hydrolyzed supplements were present in only one-fourth the concentration of the unhydrolyzed, and for absolute comparison the effects produced are, therefore, roughly 4 times larger than the figures presented (see table 3). Starch, agar, and beef extract showed no antibacillin activity either before or after hydrolysis. Peptone and egg albumin had some activity in the unhydrolyzed state and there was about a 4-fold increase through hydrolysis. Gelatin, casein, cotton seed meal, and wheat bran had no activity unhydrolyzed, but formed antibacillin on hydrolysis, the

effect decreasing in the order named. It is of particular interest to note that the activity was greatest in the two materials which are essentially pure proteins, namely, gelatin and casein. It would seem that antibacillin is liberated on hydrolysis of proteins, and further, as will be seen later, it appears to be a split product of low molecular weight, probably a peptide. Antibacillin is present in free or bound form in all the biological systems tested, including animal organs, plants, and microorganisms.

A mixture of thiamine, riboflavin, nicotinic acid, folic acid, pyridoxamine,

TABLE 2

Effect of acid hydrolysis on antibacillin activity of natural materials

SUPPLEMENT TO GLUCOSE ASPARAGINE AGAR	DILUTION OF BACILLIN CONCENTRATE INHIBITING E. COLI WITH	
	Unhydrolyzed supplement, 0.5 per cent	Hydrolyzed supplement, 0.125 per cent
None	7,680-10,240	7,680-10,240
Starch	7,680	7,680
Agar	7,680	7,680
Gelatin	5,120-7,680	480-640
Casein.....	>7,680	640-980
Peptone.....	1,920-2,560	1,280-1,920
Beef extract.....	>7,680	7,680
Egg albumin	1,280-1,920	1,280-1,920
Wheat bran	>7,680	5,120-7,680
Cotton seed meal.....	>7,680	2,840-5,120
Milk (solids basis).....	<320	1,280-1,920

TABLE 3

Relation between concentration of natural material and its antibacillin activity

CONCENTRATION OF N-Z CASE*	DILUTION OF BACILLIN CONCENTRATE INHIBITING E. COLI
Per cent	
0	2,560
0.25	640-1,280
0.5	320
1.0	160-320
2.0	80

* As supplement to glucose asparagine medium.

pantothenic acid, and biotin had no antibacillin activity. The antibacillin activity of materials containing this substance is in fairly direct proportion to their concentration, as seen in table 3. In this experiment, for each 2-fold increase of supplement, the *E. coli* assay value was cut approximately in half, i.e., the antibacillin activity increased 2-fold.

Gelatin, one of the richest sources of antibacillin and an essentially pure raw material, was used in the initial purification work reported in this paper. Three different brands of gelatin, Silver Label, Difco, and Knox, all contained about

the same amount of antibacillin after acid hydrolysis under identical conditions. A reconstructed gelatin hydrolyzate prepared from pure amino acids in concentrations simulating the composition of gelatin was without antibacillin activity, as was the same composite treated with H_2SO_4 in a manner corresponding to that employed in the hydrolysis of whole gelatin. These experiments would indicate that antibacillin is not one or more pure amino acids nor substances apt to be produced from amino acids by heating at 120 C for 1 hour with 2N H_2SO_4 .

The following experiment indicates that antibacillin exists in a bound form in gelatin and is liberated during hydrolysis. The antibacillin content of gelatin was followed as a function of time of hydrolysis at 120 C with 6.7N H_2SO_4 . The results showed that the antibacillin content was highest after 1 hour of hydrolysis, after which period it declined markedly, suggesting that the compound or compounds having antibacillin activity were further hydrolyzed or changed, with a consequent loss of activity. Almost all the activity was de-

TABLE 4
Effect of strength of acid on liberation of antibacillin from gelatin

CONC. OF H_2SO_4 USED DURING HYDROLYSIS*	DILUTION OF BACILLIN CONCENTRATE INHIBITING <i>E. COLI</i> †
No supplement.....	10,240
No acid (unhydrolyzed gelatin).....	10,240
0.25N.....	2,560-5,120
0.5N.....	1,280-2,560
1N.....	320-640
2N.....	160
4N.....	160-320
6.7N.....	160-320

* For 1 hour at 120 C.

† Concentration of hydrolyzate = 0.2 per cent in all cases. Glucose asparagine agar was the assay medium.

stroyed after 6 hours. Antibacillin liberation is proportional to the strength of H_2SO_4 up to 2 normal (table 4).

Liberation by enzymatic hydrolysis. Digestion of gelatin with the proteolytic enzymes, papain and trypsin, liberated antibacillin. The trypsin digest of a 1 per cent gelatin solution after 24 hours had as much antibacillin activity as acid-hydrolyzed gelatin.

Hydrogen sulfide and sulfur compounds. Precipitation studies with heavy metals during the purification of bacillin itself always resulted in complete loss of antibacterial activity when the excess metal cations were removed with H_2S , and it was found that bubbling H_2S through a solution of bacillin resulted in total loss of activity. With the possibility in mind that antibacillin might be a sulfur-containing compound, various pure sulfur compounds were tested for their ability to neutralize the antibacterial effect of bacillin. Cysteine, when present in 0.2 per cent concentration, had a marked effect, reducing the dilution

of the bacillin concentrate required for inhibition of *E. coli* from between 1,280 and 2,560 to 160. None of the other pure amino acids were effective even at concentrations as high as 1.0 per cent.

Further consideration of the cysteine effect led to the belief that it might be related to the destructive action of H_2S on bacillin, as mentioned earlier. Confirmation of this was obtained by the discovery that cysteine was active only when autoclaved in the medium under neutral or alkaline conditions (table 5) and that its activity could be ascribed to the liberation of H_2S , which could easily be detected by its odor. Cysteine sterilized by Seitz filtration so that H_2S was not liberated did not destroy bacillin, nor did the more stable sulfur compounds cystine, methionine, and Na-thioglycolate despite autoclaving, although the last named showed small activity. The failure of cysteine to inactivate bacillin indicates a mechanism different from that of cysteine on other antibiotics recently reported (Cavallito and Bailey, 1944; Geiger and Conn, 1945; Chow and McKee, 1945).

TABLE 5
Antibacillin activity of some sulfur compounds

SULFUR COMPOUND*	DILUTION OF BACILLIN CONCENTRATE INHIBITING <i>E. COLI</i>
No supplement.....	5,120-10,240
Cysteine, autoclaved pH 7.0.....	160-320
Cysteine, autoclaved pH 11.0.....	40-80
Cysteine, Seitz-filtered.....	2,560-5,120
Cystine, autoclaved pH 7.0.....	2,560-5,120
Methionine, autoclaved pH 7.0.....	2,560-5,120
Na-thioglycolate, autoclaved pH 7.0..	1,280-2,560
Peptidase (1%), autoclaved.....	160-320

* 0.24 per cent added to glucose asparagine medium.

Further proof that the antibacillin activity of gelatin and casein hydrolyzates is due to a factor or factors other than a sulfhydryl-containing substance was adduced from an experiment in which the activities of the hydrolyzates were at least 3 times greater than an amount of H_2S equivalent to the sulfur content of the hydrolyzates. Actually most of the sulfur in the hydrolyzate consists of methionine and cystine, already shown to be inactive. Hence, the antibacillin activity of gelatin is far in excess of that attributable to its sulfur content.

Reducing conditions as developed by addition of reduced iron to a synthetic medium and by incubation in deep tubes of broth did not affect the antibacillin activity of the hydrolyzates. No differences in antibacillin activity were observed in media adjusted to pH 6.0, 7.0, and 8.0.

Effect of inorganic salts on bacillin activity; organic nature of antibacillin. Inorganic salts are able to depress the antibacterial activity of bacillin (table 6). Possibly a portion of the activities of the various organic materials listed in tables 1 and 2 may be ascribed to their salt content, but, in every case tested so

far, the final salt concentration in media supplemented with 1 per cent of the organic materials was far too low to account for the full activity of the materials, indicating the presence of an organic inhibitor. For example, as shown in table 6, the antibacillin activity of gelatin hydrolyzate is due to its content of an organic inhibitor (antibacillin) and not to the minerals contained in it. The salt effect is nonspecific, a number of different ones producing the effect, which is very marked at concentrations of 1 per cent or more. Three per cent NH_4Cl in the medium abolished entirely for *E. coli* the inhibitory action of a bacillin preparation which, in the absence of the salt, could still inhibit this organism at a dilution of 1 to 5,120. As seen in table 6, as little as 0.1 per cent salt (NH_4Cl) reduced the bacillin titer to one-fourth that of the control.

To rule out the effect of NH_4 ion in the gelatin hydrolyzate, a portion was made alkaline with $\text{Ba}(\text{OH})_2$ and boiled to expel NH_3 , then neutralized with

TABLE 6
*Effect of salts on bacillin activity**

SALT ADDED	DILUTION OF BACILLIN CONCENTRATE INHIBITING <i>E. COLI</i>
None.....	5,120-10,240
0.1% NH_4Cl	1,280
0.3% NH_4Cl	640-1,280
1.0% NH_4Cl	320
3.0% NH_4Cl	<20
1.5% KCl	160-320
2.0% KCl	80-160
3.0% KCl	40-80
1.6% NaCl	160
3.1% MgCl_2	320-640
1.6% Na_2SO_4	320-640
1% hydrolyzed gelatin	160-320
Ash from 1% hydrolyzed gelatin	1,280-2,560

* Glucose asparagine agar.

H_2SO_4 , and the BaSO_4 removed. The antibacterial activity was unchanged, indicating that the antibacillin action of hydrolyzed gelatin almost certainly is not due to inorganic material. Final confirmation of the organic nature of antibacillin was provided by the complete destruction of activity in gelatin hydrolyzate by treatment with H_2O_2 .

Competitive nature of the bacillin-antibacillin effect. Some attention was given to the nature of the antibacillin effect, and evidence was obtained for a competitive action between bacillin and antibacillin. Reversibility proportional to varying concentrations as described in table 3 is presumptive evidence of a competitive action. The following experiment shows that antibacillin does not destroy bacillin, nor does it combine with it to render the bacillin inactive, as antithiamine inactivates thiamine and avidin inhibits biotin. A bacillin concentrate which completely inhibited *E. coli* at a dilution of 51,200 was divided

into two equal portions. One portion was kept for 1 hour in contact with an aqueous solution of an antibacillin concentrate prepared from hydrolyzed gelatin as described below. A sample of the mixture taken for test showed that the antibacillin had counteracted entirely the inhibitory powers of the bacillin. The second portion of bacillin was used as an untreated control. The procedure previously described for the isolation of bacillin from broth (see the preceding paper) was now applied to the bacillin-antibacillin mixture and to the control bacillin. The solutions were treated with 2 per cent norite and the norite eluted with 90 per cent ethanol. The eluate was evaporated *in vacuo*, and an aqueous solution of the residue was tested for bacillin activity. The data showed that active bacillin was recovered from the inactive mixture, and that, moreover, the yield was essentially the same as that from the control bacillin carried through the same isolation procedure. Thus, bacillin is not destroyed by antibacillin, and it appears unlikely that it makes a chemical combination with bacillin which renders the latter inactive, unless one assumes that the mild reisolation procedure destroyed the combination and liberated the bacillin. More probable is a competitive action between the two in the metabolism of the bacteria. In the above experiment full antibacillin activity was recovered from the solution after charcoal adsorption.

Properties of antibacillin; purification studies. Antibacillin dialyzes completely through a cellophane membrane in running water within 3 hours.

No specific inorganic precipitant was found for the bacillin inhibitor, although some activity was present in the oil layer that separated out upon the addition of 10 volumes of acetone to an aqueous solution. Some impurities, but no antibacillin activity, were precipitated by phosphotungstic acid, lead acetate at different pH's, $\text{Ba}(\text{OH})_2 + \text{CO}_2$ (forming amino acid carbamates), cupric carbonate, cadmium chloride, and ammonium rhodanilate.

Antibacillin is soluble in methanol, ethanol, and isopropanol. Preliminary extraction trials by various immiscible organic solvents were unsuccessful, but it was found that the active component could be extracted from aqueous solution with *n*-butanol upon prolonged continuous extraction *in vacuo*. The results of a typical fractionation experiment employing butanol follow: A 20-fold weight concentration was obtained which resulted in an over-all 10-fold purification, since only about half the activity was recovered in the butanol. The water residue from the extraction had about one-eighth of its original antibacillin activity, indicating that the active fraction is extracted very slowly. About 80 per cent of the material in the butanol extract consisted of amino acids or peptides, as measured by formol titration. (The figure 120 was arbitrarily adopted as the average molecular weight of the amino acids in gelatin.) The solid material which settled out in the butanol during the extraction was practically inactive. This fraction consisted mainly of proline. The active fraction could be separated from the butanol by the addition of 5 volumes of ether. About a 50 per cent increase in potency was achieved in this way. Attempts toward purification by fractional precipitation from butanol with acetone resulted in complete loss of activity. Several experiments have demonstrated this

inactivation of antibacillin in the presence of acetone. Passing through an aluminum oxide column the yellowish water solution made by shaking the butanol extract with 3 volumes of water resulted in the retention on the column of about 50 per cent of the inert impurities, including the yellow coloring matter, but the antibacillin was not adsorbed. However, no purification was achieved by chromatographing an aqueous solution of the active ether precipitate from the butanol extract. Fractional precipitation from water with HgCl_2 and from butanol by methyl-*n*-hexanol (oily ppt.) yielded fractions, all of which had approximately the same specific antibacillin activity.

At this point, nitrogen analysis was made of the ether-precipitated material. Total N was determined by micro-Kjeldahl, amino N by formol titration, and the specific amino acids by microbiological assay. Total N was 10.9 per cent, of which 10.2 (93.6 per cent of the total) was contained in free amino groups. At least 12 per cent of the material was impurities in the form of the amino acids leucine, isoleucine, and valine, each amounting to 4 per cent of the total precipitate analyzed. This combination of amino acids had no antibacillin activity. Cystine, methionine, tryptophane, glutamic acid, and threonine were absent. Prolonged acid hydrolysis of the preparation did not release any of these amino acids, nor increase the amount of valine, leucine, or isoleucine, thus indicating that these eight amino acids were not present in peptide form.

The observation that antibacillin solutions usually lost activity when they became contaminated with bacteria led to experiments which showed that rapid destruction of antibacillin occurred in cell suspensions of various bacteria, even when growth was prevented by toluene. *E. coli* was particularly active in this respect.

At this point it was found that antibacillin occurs in a number of different microbial cells, including bacteria, yeasts, molds, and actinomycetes. The bacterial cells tested, *E. coli*, *Salmonella paratyphi*, *Salmonella schottmuelleri*, *Bacillus subtilis*, and *Staphylococcus aureus*, contained no measurable free antibacillin, but antibacillin was liberated upon acid hydrolysis. When grown on organic media (nutrient broth or corn steep liquor), the mycelium of *Penicillium notatum* and *Streptomyces griseus* contained an appreciable amount of bacillin inhibitor in a free state, but only bound antibacillin was obtained on synthetic media.

Concentrates from other sources. Since a large proportion of the hydrolyzed gelatin concentrate consisted of inactive amino acids, a search was made for raw materials which contain bacillin inhibitor in a free state, thus obviating the need for hydrolysis. Three sources, milk (fresh or dried), *Penicillium notatum* mycelium, and *Streptomyces griseus* mycelium proved to be such substances.

In a typical experiment with milk, 2.6 mg of spray-dried skimmed milk per ml of glucose asparagine agar reduced the antibacterial titer of a bacillin concentrate from 50,000 to 750 units per ml. Soxhlet extraction with isopropanol resulted in an over-all purification of 30-fold, and the major impurity in the extract was lactose, amounting to 40 per cent of the total.

The *Penicillium* mold material yielded the most active concentrates of antibacillin yet obtained. A modified purification procedure was necessary with this substrate. The ash content was a major impurity. Amberlite resins removed ash as well as a considerable fraction of the impurities. It was found that antibacillin from these cell extracts could be adsorbed by high concentrations of norite. Table 7 summarizes the purification of the *P. notatum* cell material. For extraction, 2.4 kilos of moist pressed mycelium were autoclaved with water at 120 C for 15 minutes, yielding, after filtration, 10 liters of yellowish cell extract. This extract was passed through Amberlite IR1 (cationic exchange) and Amberlite IR4 (anionic exchange) resins to remove salts. The filtrate was treated with norite; 2 kilos were required for complete adsorption of the antibacillin from 10 liters of extract. Most of the recoverable antibacillin (42 per cent) was obtained in the first elution with 3.5 liters of 95 per cent ethanol, and this was 2 and 3

TABLE 7
Purification of antibacillin in *P. notatum* cell extracts

PREPARATION	WEIGHT RECOVERY	POTENCY*	OVER-ALL RECOVERY
	g	μg/ml	per cent
Cell extract.	213	5.5-8.0	
Amberlite IR1-IR4 filtrate. . .	65	2-2.8	84
Norite unadsorbed	9.75	22	1
1st ethanol eluate	16.2	1.0	42
2nd ethanol eluate	6.65	2.0	9
3rd ethanol eluate	4.69	2.8	4
Soluble portion of concentrate from 1st eluate after addition of 4 vol. 98 per cent ethanol	10.4	0.7	39
Insoluble portion of above . .	5.4	4.0	8

* Quantity required to counteract 80 per cent of the antibacterial activity of a bacillin concentrate against *E. coli* in glucose asparagine agar.

times more potent, respectively, on a solids basis, than two further successive eluates with the same amount of alcohol.

The first eluate was concentrated *in vacuo* to about 140 ml, at which point about one-third of the solids (low activity) could be precipitated by the addition of 4 volumes of 98 per cent ethanol, full antibacillin activity remaining in solution. Additional inactive material settled out in crystalline form during storage for a few days in the refrigerator. The crystalline material is unidentified but was proved not to be tyrosine. Of the total solids in the solution containing the antibacillin, 19 per cent consisted of amino acids (120 average mol wt) as determined by formol titration. Destruction of one-third of the amino N by nitrous acid did not reduce the antibacillin activity within the limits of the test. Of the most active concentrate prepared by this procedure, 0.25 μg per ml was sufficient to counteract 80 per cent of the antibacillin activity of bacillin against *E. coli* in glucose asparagine agar.

DISCUSSION

Antibacillin is a naturally occurring competitive inhibitor for an antibiotic substance. Its universal occurrence in biological systems makes it likely that this substance plays a vital rôle in metabolism. Of especial interest is the speculation that bacillin may be effective against sensitive bacteria because of its competitive action with antibacillin in the metabolism of these cells.

All evidence points to antibacillin being a peptide or a mixture of peptides. Its solubility properties, its liberation from proteins by acid or enzymatic digestion, and its stability characteristics are consistent with this. It would be, thus, one of the few known instances in which peptides per se are active biologically.

SUMMARY

Naturally occurring complex organic materials contain an organic substance, herein named "antibacillin," which counteracts the antibacterial action of the antibiotic bacillin. Inorganic salts and H_2S also have this property. Antibacillin is liberated on hydrolysis of gelatin or casein. It does not destroy or combine with bacillin but probably acts competitively with the latter for vital systems in susceptible bacteria. The chemical properties of antibacillin are given as well as details for the preparation of concentrates of the active fraction from gelatin and from *Penicillium notatum* mycelium.

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A GROWTH FACTOR IN CERTAIN VEGETABLE JUICES¹

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It has been known for some time that certain microorganisms require the presence of tomato juice to enhance and in some cases promote maximum growth. As a survey of the literature indicates little of a specific nature regarding the growth-stimulating activity of certain vegetable juices, it is the purpose of this investigation to study the nature of this factor and its possible relation to other known growth accessory substances.

Studies were made on several different basal media and assay organisms of lactic acid types to determine which might be the best adapted to the investigation.

Lactobacillus fermentum (L36) was selected as the test assay organism for the unknown growth factor, as it gave consistently the greatest spread of titratable acidity in the basal medium with and without tomato serum.

As a result of studies on basal media, the following medium was adopted for the study of the growth factor:

Tryptone	1 g
Yeast extract	1 g
Fructose	10 g
Glucose	10 g
Thiamine	0.2 μ g per ml
Inorganic salt solutions (A and B)*	15 ml each
Distilled water	970 ml
H-ion concentration	pH 6.8-7.0

*A = K_2HPO_4 , 25 g; KH_2PO_4 , 25 g; distilled water, 250 ml.

B = $MgSO_4 \cdot 7H_2O$, 10 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 4H_2O$, 0.5 g; distilled water, 250 ml.

Thiamine was added to the medium as it was the only known growth factor that was a partial substitute for tomato serum, which was made by filtering tomato juice through fine filter paper. This level of thiamine was chosen to assure an adequate amount of the vitamin, since the thiamine curve started to level off when 0.1 to 0.2 μ g per ml were added to the basal medium. The reduction of the tryptone and yeast extract to 0.1 per cent of each per liter reduced the thiamine in these ingredients to an insignificant amount, and did not affect the maximum level of acidity produced in the presence of tomato serum.

Other substitutions for tomato serum were attempted using riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, *p*-aminobenzoic acid, inositol,

¹ Journal Paper No. 639, New York State Agricultural Experimental Station, Geneva, N. Y., June 9, 1945.

choline, an eluate factor (folic acid B₁₀ and B₁₁), vitamins A, E, C, and K, arginine, leucine, lysine, tyrosine, tryptophane, cystine, glutamic acid, thallium chloride, alanine, asparagine, glycine, histidine, xanthine, methionine, adenine, guanine, uracil, cytosine, isocytosine, uric acid, citric acid, malic acid, and orotic acid. Certain combinations of vitamins, pyrimidines, amino acids, and organic acids were also studied. None of these known growth factors with the exception of thiamine enhanced the growth of L36, as compared with the increased growth produced when tomato serum was added. No additional effect was noted when substitutions were made in the presence and absence of thiamine. Without thiamine no titration curve was obtained. When thiamine was present with the other known growth factors, only the thiamine curve was noted.

TABLE 1

Effect of heat upon growth-promoting properties of thiamine and tomato serum as reflected by acid production by Lactobacillus fermentum (L36)

H-ION CONCENTRATION PH	TIME OF HEATING	THIAMINE		TOMATO SERUM		"T" FACTOR
		Maximum N/10 acid produced	Thiamine present*	Maximum N/10 acid produced	Thiamine present	Amount in terms of N/10 acid†
	<i>minutes</i>					
3.0	20	2.4	0.0	4.2		2.7
6.9	20	1.0		4.4		2.2
10.0	20	1.0		2.6		1.9
3.0	120	2.5		4.7		3.8
6.9	120	1.0	5.0	4.1		2.7
10.0	120	0.9		2.6		2.3
7.0	Unheated	2.8	99.0	4.8	41.0	2.4

* μ g per 100 ml determined by thiochrome method.

† Amount of N/10 acid produced in addition to N/10 acid produced at maximum level of thiamine.

That the tomato serum ("T" factor) may be closely related to or act in conjunction with the thiamine is possible. However, certain basic characteristics, particularly heat resistance, indicate its separate identity. In a series of assays (table 1) in which thiamine was adjusted to pH 3.0, 6.9, and 10.0 and heated for 20 minutes and 2 hours at 250 F, and then added to the medium and sterilized for 10 minutes at 250 F, it was indicated that at H-ion concentrations of pH 6.9 and 10.0 thiamine was destroyed. Tomato serum when treated at these H-ion concentrations in the same manner indicated (table 1) that the "T" factor is resistant at this temperature. It may be possible that in the presence of tomato serum thiamine is more resistant in the acid range.

To determine whether thiamine split products might be effective, thiamine solutions were treated with sulfite, but no activity remained. Treatment of a thiamine solution with alkaline ferricyanide and extraction of the thiochrome with isobutanol also completely inactivated the thiamine. The pyrophosphate ester, cocarboxylase, had no growth-promoting action on the test organism.

The index of growth at the various thiamine and tomato serum levels has been the amount of acid produced (figure 1). At a 5 per cent level of tomato serum the titratable acidity and H-ion concentration were at a maximum. The number of cells in the assays of the tomato serum or thiamine increased as the acidity increased. At the maximum levels the number of cells present in the thiamine and the tomato serum assay cultures was found to be in direct ratio to the amount of acid produced.

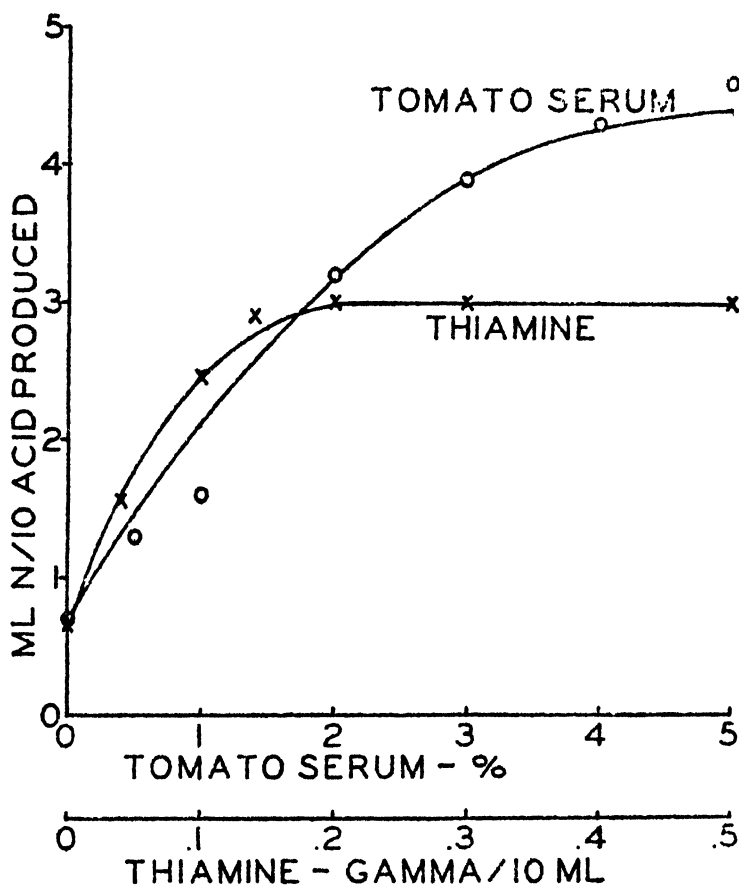


FIG. 1. RELATIVE GROWTH-PROMOTING PROPERTIES OF TOMATO SERUM AND THIAMINE AS REFLECTED BY ACID PRODUCTION BY *LACTOBACILLUS FERMENTUM* (L36)

Studies on other materials indicated that this "T" factor may also be present in liver, string beans, carrots, beets, onions, cabbage, peppers, spinach, and orange juice.

DISCUSSION

It appears that there is present in tomato juice and certain other vegetables an unidentified growth-promoting substance, as indicated by the stimulation of

acid production by certain *Lactobacillus* cultures. This growth-promoting substance is not replaceable by any of the known growth accessory substances with the exception of thiamine. By the use of the test culture (L36) now available, it is indicated that thiamine will in part replace (figure 1) or promote partial growth of this test organism. From a study of the bioassay curves, tomato serum will produce with this organism a maximum of approximately 4.8 ml of N/10 acid.

There are three possible interpretations which might be suggested on the relation of this "T" factor to thiamine: (a) the "T" factor may be entirely separate and distinct from thiamine; (b) tomato juice may contain a factor which will act in conjunction with thiamine to produce an increased activity of the test organism; (c) the "T" factor in tomato and certain other vegetable juices may be due to thiamine present in some particular available form which is not detectable by known chemical methods, such as the thiochrome method for the determination of thiamine. If this latter instance is true, the maximum acidity as produced by tomato and other vegetable juices may be brought about by the presence of thiamine in some state which is more available to assay organisms.

The evidence, however, indicates on the basis of present information, particularly as it relates to the heat-labile characteristics of thiamine, that tomato and other vegetable juices contain a growth accessory factor which may be entirely distinct from previously known substances and which has not as yet been isolated and identified.

CONCLUSIONS

There is present in tomato and certain other vegetable juices an unidentified growth accessory substance. This "T" factor may act in conjunction with thiamine, but its heat-stable properties indicate that it is distinct from thiamine. The "T" factor was also found in liver, string beans, carrots, beets, onions, cabbage, peppers, spinach, and orange juice.

METABOLIC CHANGES IN SUBMERGED PENICILLIN FERMENTATIONS ON SYNTHETIC MEDIA^{1, 2}

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Earlier reports from these laboratories dealt with the chemical changes during the submerged growth of penicillin-producing molds in corn steep media (Koffler, Emerson, Perlman, and Burris, 1945; Koffler, Knight, Emerson, and Burris, 1945). This paper describes the metabolism of *Penicillium chrysogenum* X-1612 during growth on certain synthetic media.

METHODS

The Production of Penicillin in Shake Flask Fermentations

Details about the shake flask method of penicillin production have been given in earlier papers; the same procedures were followed in this investigation, with the following exceptions.

Preparation of inoculum. The medium used for the growth of the spore inoculum had the following composition:

Sugar beet molasses.	5.00 g
Peptone.	5.00 g
NaCl.	4.00 g
KH ₂ PO ₄	0.10 g
MgSO ₄ ·7H ₂ O.	0.05 g

Distilled water to make one liter

The pH of the medium was not adjusted.

Media. Four synthetic media were found useful during this study. Medium A was essentially the one used by D. Perlman, formerly of these laboratories, and had the following composition: lactose (cp), 20.00 g; dextrin, 5.00 g; glacial acetic acid, 4.00 g; NH₄NO₃, 6.00 g; KH₂PO₄, 1.50 g; MgSO₄·7H₂O, 0.25 g; ZnSO₄·7H₂O, 0.04 g; distilled water to make 1 L. Medium B was described by Knight and Frazier (1945), who observed that ashed corn steep, when added to medium A, enhanced penicillin yields. The gray corn steep ash was obtained by heating corn steep solids (University of Wisconsin no. 71, purchased on July 20, 1945, from the Staley Company, Decatur, Illinois) in an electric furnace at 1,400 F for 4 to 5 hours. Five g of this ash were added to 1 L of medium A.

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² The work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Media C and D, and other modifications of the original medium A, were worked out during this investigation. Medium C is the same as medium A, but contains 4.90 g of $(\text{NH}_4)_2\text{SO}_4$ instead of 6 g of NH_4NO_3 . Medium D has the same composition as C but also contains 5.00 g of corn steep ash per L.³ The pH of each medium was adjusted with KOH to 6.3 before autoclaving. The use of phenylacetamide in synthetic media was suggested by workers of the Northern Regional Research Laboratories. Whenever the term "ash-free media" is used in this paper, it refers to substrates which do not contain corn steep ash; similarly, "ash media" contain ashed corn steep.

The Analysis of Cultures

Dry weight. Three shake flask cultures were pooled daily, starting on the third day of the fermentation, and filtered on a Buchner funnel; the filtrate was preserved for analysis of penicillin, sugar, and nitrogen compounds. The residue was washed with distilled water and dried at 100 C for 12 hours before weighing. Media B and D also contained some undissolved ash, either occluded in the mold pellets or precipitated on the bottom of the flask. To obtain the approximate estimate of vegetative growth the weight of the residual ash from uninoculated control flasks was subtracted from the weight of mycelium plus ash in inoculated flasks.

Penicillin. The culture filtrate was assayed for penicillin by the Oxford cup method with *Staphylococcus aureus* FDA 209P as the test organism (Foster and Woodruff, 1944; Schmidt and Moyer, 1944). A penicillin preparation from the Food and Drug Administration, Washington, D. C., was used as the reference standard.

pH. The pH was determined with a glass electrode immediately after filtration of the broth.

Sugars. Lactose and dextrin were hydrolyzed in 0.75 N HCl solution in the autoclave at 15 pounds' pressure for 30 minutes, and the neutralized hydrolyzate was analyzed by the method of Shaffer and Somogyi (1933). Standard titration curves were prepared with results expressed in terms of lactose.

Ammonia nitrogen. An aliquot containing 0.5 to 5.0 mg of ammonia nitrogen was made alkaline by the addition of 10 ml of phosphate-borate buffer and 3 ml of NaOH-borate buffer, as described by Pucher, Vickery, and Leavenworth (1935). The ammonia was then aerated into standard acid and titrated according to the directions of Umbreit and Bond (1936).

Amide nitrogen. The method of Pucher, Vickery, and Leavenworth (1935) was employed. After acid hydrolysis the sample was made alkaline, aerated, and titrated as for the analysis of ammonia. The results were corrected for ammonia, which was determined independently.

Nitrate nitrogen. Devarda's alloy was added to another aliquot of the broth, and the ammonia originally present together with that arising from the reduction

³ The use of $(\text{NH}_4)_2\text{SO}_4$ rather than NH_4NO_3 as the source of nitrogen avoided the interference of the nitrate ions with the nitrogen determinations described later and favored almost identical penicillin yields and metabolic pattern.

of nitrates was aerated and titrated. Corrected for ammonia, this titration gave the amount of nitrate nitrogen present in the sample. A correction for amide nitrogen which might have been released during the cold aeration was unnecessary because amide nitrogen was present only in traces.

Total organic nitrogen. This value was determined by the method of Umbreit and Bond (1936) and served to check the accuracy of the nitrogen determinations made independently on the basic and nonbasic nitrogen fractions. Since nitrates interfere with this determination, nitrate-containing samples were analyzed only after the removal of nitrates, ammonia, and amides. Media free of nitrates, however, were analyzed directly; by subtraction of the value for ammonia from the Kjeldahl nitrogen value the nitrogen content of the total organic nitrogen fraction could be found.

Amino nitrogen. Pooled samples (equivalent to 50 ml of the original fermentation broth) from which amides and ammonia had been removed were made slightly acid to bromthymol blue and concentrated to 15 or 20 ml on the steam bath. The concentrate was then transferred to a volumetric flask and made up to a volume of 25 ml. Amino nitrogen values were determined on 2-ml aliquots of this dilution by the Van Slyke nitrous acid method.

Basic and nonbasic nitrogen. An aliquot of the solution from the amino nitrogen determination was analyzed by the method of Umbreit and Wilson (1936). Before determination of the Kjeldahl nitrogen content of the phosphotungstic acid precipitate and phosphotungstic acid filtrate, nitrates were removed with the aid of Devarda's alloy, after which the Devarda alloy was filtered off.

EXPERIMENTAL

Studies of the chemical changes in submerged penicillin fermentations on corn steep media indicated a correlation between ammonia concentrations and penicillin yields. These relationships were considered significant enough to be investigated further. Since corn steep media, because of their complexity, did not seem suitable for more detailed chemical studies, these investigations were conducted on synthetic substrates which contained known sources of nitrogen.

Table 1 presents information on the penicillin yields, pH values, and ammonia levels obtained on various synthetic media. The addition of corn steep ash to medium A enhanced penicillin yields very markedly. If corn steep ash and phenylacetamide were added to this basal medium, the stimulation was even more pronounced; in fact, medium B with or without phenylacetamide favored production of penicillin as high as or higher than did the 3 per cent lactose, 3 per cent corn steep medium. Phenylacetamide, added without ash, was capable of increasing penicillin yields but to a much lesser extent than was the corn steep ash. The effect of boric acid, which was previously (Koffler, Knight, Emerson, and Burris, 1945) shown to stimulate penicillin accumulation in corn steep media, was erratic; a slight stimulation could usually be observed. The pH picture was fairly similar for all media employed.

Table 1 strikingly demonstrates that the ash-free media contained much

higher ammonia levels throughout the entire fermentation than did the media with corn steep ash. Organisms grown on media containing corn steep ash began to autolyze 1 or 2 days earlier than they did on ash-free media.

Figures 1 and 2 are representative of the data obtained by seven additional experiments and serve to contrast two diverse types of metabolism, one being characteristic for growth on media A and C, and the other for B and D, which contain corn steep ash.

Fermentation phases. The existence of three phases in the fermentation on corn steep media have been pointed out in earlier reports.⁴ Fermentations conducted on synthetic media showed an analogous phasic division. On media containing corn steep ash, the second fermentation phase was reached from 1 to 2 days sooner than on ash-free media.

Penicillin. The production of penicillin in media containing corn steep

TABLE 1
Penicillin yields on synthetic media with associated pH values and ammonia levels

MEDIUM	pH						PENICILLIN (OXFORD UNITS/ML)					AMMONIA N (MG/100 ML)							
	Days																		
	3	4	5	6	7	8	4	5	6	7	8	3	4	5	6	7	8		
A.....	7.17	7.38	7.44	7.44	7.62	7.92	41	48	48	36	11	30.9	36.7	35.8	33.3	46.8	55.3		
A + 0.05% phenylacetamide..	7.27	7.39	7.39	7.40	7.61	8.02	71	77	68	62	46	47.6	48.9	39.0	37.4	43.7	55.4		
A + 0.065% boric acid.....	7.14	7.29	7.31	7.61	7.84	7.81	41	47	44	39	15	35.3	34.3	36.4	(18.5)	46.8	53.4		
A + 0.13% boric acid.....	7.20	7.27	7.30	7.49	7.53	7.72	45	42	43	43	16	39.7	34.4	38.2	34.3	47.5	54.0		
B*	7.22	7.30	7.40	7.37	7.66	8.00	74	107	115	115	120	15.9	3.5	6.7	15.8	25.9	24.7		
B + 0.05% phenylacetamide ..	7.22	7.26	7.33	7.36	7.59	8.04	107	145	195	190	155	14.2	6.0	5.6	23.0	23.7	29.4		
B + 0.065% boric acid.....	7.24	7.22	7.38	7.46	7.71	7.96	81	101	110	117	90	18.4	2.2	6.2	19.9	24.7	19.9		
B + 0.13% boric acid	7.32	7.19	7.30	7.32	7.74	7.92	78	87	121	130	117	22.1	3.5	3.9	36.1	27.1	20.1		
3% lactose, 3% corn steep†...			7.54	7.51	7.45	7.41		71	88	83	76								

* Medium B is the same as medium A but contains in addition 0.5 per cent corn steep ash.

† This medium also contained the following salts in addition to lactose and corn steep: $MgSO_4 \cdot 7H_2O$, 0.25 g; $NaNO_3$, 3.00 g; KH_2PO_4 , 0.50 g; $ZnSO_4$, 0.025 g; water to make 1 L.

ash (media B and D) was greater throughout the entire fermentation than in ash-free substrates (media A and C). The maximum concentration was usually about doubled.

pH. Since the pH of all synthetic media was adjusted to 6.3 before autoclaving, the initial pH rise was much less conspicuous in these than in corn steep media. Hydrogen ion concentrations favorable for penicillin production were reached by the third day. The drop in pH preceding a second rise—characteristic for corn steep media—was usually insignificantly small in these fermenta-

‘The following are some of the distinguishing characteristics of these stages:

Phase 1—Slight production of penicillin, rise in pH, rapid growth of mycelium, slow utilization of sugars.

Phase 2—Maximum rate of penicillin production, pH plateau or drop in pH, slow mycelial growth, rapid sugar utilization.

Phase 3—Decrease in the concentration of penicillin, rise in pH, decrease in mycelial weight, slow utilization of sugars.

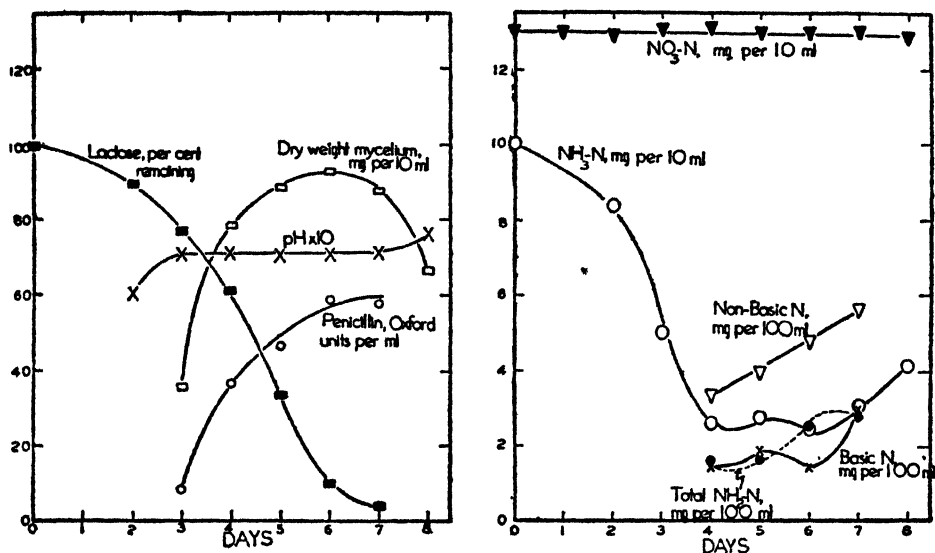


FIG. 1. CHEMICAL CHANGES TYPICAL OF SHAKE FLASK FERMENTATIONS ON MEDIA WITHOUT CORN STEEP ASH

The metabolic changes in medium A, which contains NH_4NO_3 , are nearly identical with those in medium C, which contains $(\text{NH}_4)_2\text{SO}_4$ as the source of nitrogen.

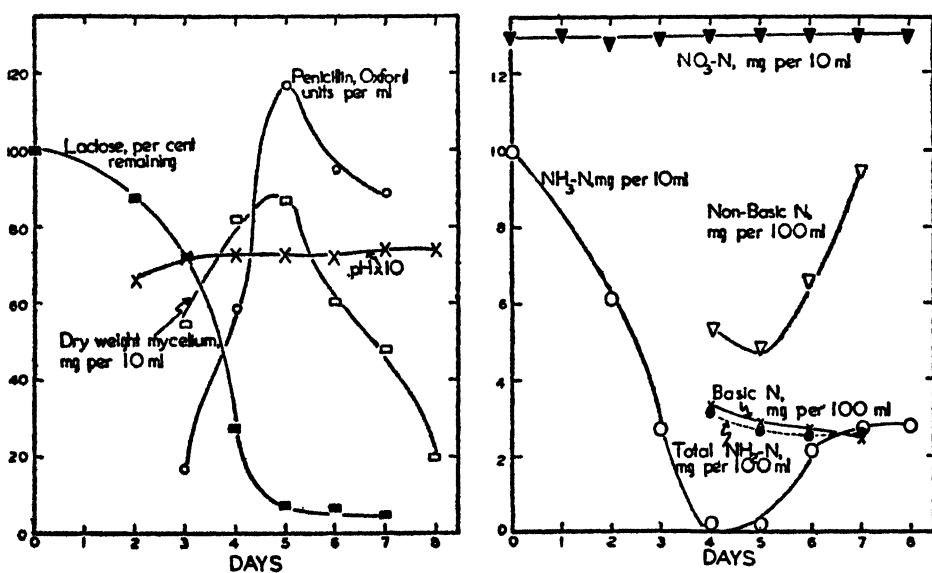


FIG. 2. CHEMICAL CHANGES TYPICAL OF SHAKE FLASK FERMENTATIONS ON MEDIA CONTAINING CORN STEEP ASH

The metabolic changes in medium B, which contains NH_4NO_3 , are nearly identical with those in medium D, which contains $(\text{NH}_4)_2\text{SO}_4$ as the source of nitrogen.

tions. The pH rose slowly throughout the entire fermentation, maintaining a reaction which is usually considered satisfactory for penicillin production. In almost all cases the pH values for fermentations carried out on ash media did not differ significantly from those on ash-free media.

Dry weight of mycelium. Media containing corn steep ash supported more abundant growth during the first 4 days than did ash-free media. Although the organism reached maximum development 1 or 2 days earlier on the former, its maximum weight usually was lower than on the latter. Autolysis of the mycelium was more rapid in the ash medium.

Carbohydrates. The utilization of sugars closely paralleled the development of the mycelium. Consequently, sugars were exhausted more rapidly in media B and D than in A and C.

Ammonia nitrogen. Organisms grown on ash media made much more active use of ammonia than did cultures on ash-free media. On both media the lowest level of ammonia was found on the fourth day. Whereas the organisms given the mineral elements of corn steep utilized ammonia to exhaustion, those not furnished with corn steep ash consumed less than two-thirds of the ammonia supply. Interestingly enough, the latter ceased to utilize ammonia 1 to 2 days before they had attained full development, although there was still a high level of ammonia left. Organisms grown on media with ash, however, had completely exhausted the ammonia, as well as the sugars, by the time they were fully grown, and they disintegrated immediately thereafter. The molds grown on ash not only showed active synthetic activity but also autolyzed more completely and rapidly than mold cultures without ash. Ammonia was the most abundant nitrogen compound determined during autolysis.

Nitrate nitrogen. Nitrates, if present in addition to sufficient quantities of ammonia (i.e., media A and B), were scarcely utilized. Experiments with nitrates as the sole nitrogen source have not been made.

Amide nitrogen. Amide nitrogen could be detected only in traces. The amounts determined always were within the limits of experimental error.

Humin nitrogen. The precipitate formed during the acid hydrolysis of amides usually contained less than 1 mg and never more than 1.5 mg of Kjeldahl nitrogen per 100 ml of fermentation broth.

Amino nitrogen. The amino nitrogen was excreted in very low concentrations. It is plausible that amino compounds are deaminated as soon as excreted, this deamination giving rise to ammonia and carbon residues. It is difficult to evaluate the differences in amino nitrogen concentration found under different states of nutrition because of the small quantities involved. Whereas the concentration of amino nitrogen increased during autolysis in ash-free media, it decreased in ash media; however, the ash-fed mold excreted slightly more amino nitrogen.

Basic nitrogen. The amounts of basic nitrogen detected during the autolytic phase were small. Organisms grown on ash-free media excreted progressively larger amounts of basic nitrogen compounds with time, but liquors containing ash showed only a small increase of basic nitrogen compounds, or even a decrease.

Nonbasic nitrogen. Next to ammonia the nonbasic nitrogen compounds were

found in largest quantities. On both types of substrate the concentration of these compounds increased during autolysis but more significantly during the disintegration of the ash-fed mold. The amino nitrogen values accounted only incompletely for the nonbasic nitrogen compounds.

The effect of boric acid on metabolism. A single experiment, in addition to the experiments presented in table 1 and figures 1 and 2, was conducted to study the effect of boric acid on mold metabolism. The physiological picture obtained when *P. chrysogenum* X-1612 was grown on medium A to which 0.065 per cent boric acid had been added was identical to the one characteristic for medium A, with the following exceptions: (1) This experiment indicated a significant stimulation of penicillin yields (i.e., the maximum level for medium A was 42 Oxford units per 100 ml, but for medium A plus boric acid it was 59). (2) Maximum weight was obtained on the sixth day rather than on the fifth day. (3) On and after the fourth day mycelial weights were lower on the boron medium than on the boron-free medium. (4) The nonbasic fraction was slightly smaller on the fourth, fifth, and sixth days than the basic nitrogen fraction.

SUMMARY

The addition of the ash from corn steep to a medium consisting of lactose, dextrin, and mineral salts caused a remarkable increase in penicillin yields in shake flask fermentations. If phenylacetamide also was added, yields of penicillin were even greater and were as high as or higher than those previously obtained on corn steep media. Boric acid may slightly stimulate the formation of penicillin. Corn steep ash had a distinct accelerating influence on mold metabolism. *Penicillium chrysogenum* X-1612 when grown on ash media utilized sugars and ammonia more rapidly and to a much greater extent than did the same mold on the medium without added ash.

Not only the synthetic processes but also the decomposition was conspicuously accelerated by the addition of corn steep ash; the rate at which the ammonia, nonbasic nitrogen, and basic nitrogen fractions were excreted was considerably faster in ash media than without added ash. During the disintegration of the mold, ammonia was by far the most abundant nitrogen compound determined. The concentration of amino nitrogen and basic nitrogen compounds was low and amide nitrogen was either absent or present only in traces. The compounds of the nonbasic fraction, however, were excreted in slightly greater amounts, and more rapidly in the ash-treated fermentations than they were in ash-free fermentations. Ammonia levels always were considerably lower when corn steep ash was added to the media, and in such media ammonia was almost completely exhausted during the growth of the mold.

ACKNOWLEDGMENT

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THE EFFECT OF DESICCATION ON THE ACTIVITY AND MOISTURE CONTENT OF BAKERS' YEAST¹

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Little information is available on the effect of desiccation in the retention of metabolic activity by microorganisms. There is also a lack of information on the amount of water lost by microbial cells on drying to equilibrium with various water vapor pressures. This note reports experiments in which these variables were investigated for commercial bakers' yeast.

EXPERIMENTAL

Duplicate 0.5-gram samples of moist commercial bakers' yeast were placed in open weighing bottles over 25 grams of sulfuric acid water solutions in individual desiccators. The concentration of these solutions ranged from 25 to 94 per cent sulfuric acid, giving water vapor pressures from 26 to 0.0016 mm mercury (Blake and Greenewalt, 1928). The water removed from the yeast was taken into account in calculating the acid concentration. The samples were stored at 30 C until constant weight (to 0.001 g) was reached in all samples. This required 6 to 7 days. One of the duplicate desiccated samples was then dried for 24 hours at 110 C to obtain oven-dry weight. The other sample was used for the metabolic study.

Anaerobic carbon dioxide production was determined at 30 C by the Warburg technique. The medium employed was:

Sodium acetate, acetic acid buffer, pH 4.7.....	0.05 M
KH ₂ PO ₄	0.010
Glucose.....	0.044
Mg as sulfate.....	0.0013
Mn as sulfate.....	0.0006
Thiamine.....	0.0005

Three ml of this medium and approximately 2 mg of yeast (dry basis) were used in each flask. Anaerobic conditions were obtained by a 5-minute nitrogen wash. After a 40-minute induction period, the rate of carbon dioxide output remained constant and was measured for the additional 60-minute period used in the data.

¹Supported in part by a grant from the Red Star Yeast and Products Company, Milwaukee. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

RESULTS AND DISCUSSION

The data from three drying experiments are summarized in figure 1. The moisture content is plotted as grams of water retained per gram of oven-dry yeast. The vapor pressures are plotted logarithmically as mm of mercury. All calculations and data are based on the assumption that the 24-hour drying at 110 C completely removes the water from the yeast. From the graph it is apparent that all the water of the yeast was not removed by desiccation, even over concentrated sulfuric acid. The curve of water retained with reference to vapor pressure may be divided into three portions. First, marked loss of water occurred from 26 to about 12 mm vapor pressure, resulting in the retention

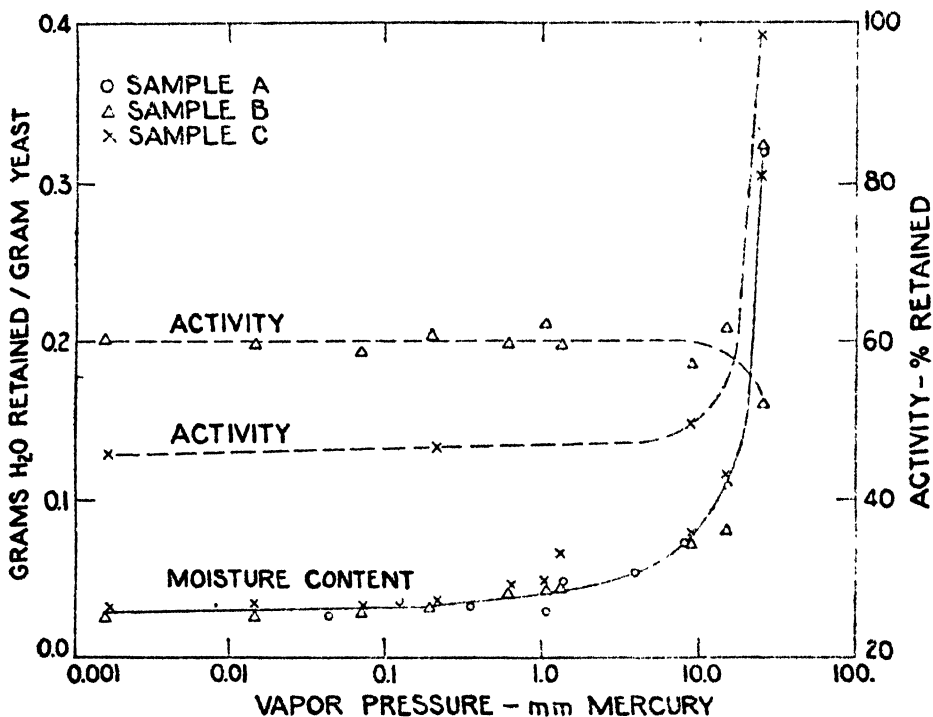


FIG. 1. MOISTURE CONTENT AND METABOLIC ACTIVITY OF YEAST DRIED AT VARIOUS WATER VAPOR PRESSURES

of 0.1 gram of water per gram of yeast, a level comparable to that of commercial active dried bakers' yeast. Second, from 12 to 0.1 mm vapor pressure a gradual decrease in water retained occurred until a level of 0.03 gram of water per gram of yeast was reached at a vapor pressure of about 0.1 mm mercury. Third, from this point to drying over concentrated acid at a vapor pressure of 0.0016 mm, no further loss of water occurred. The remainder of the water was apparently very firmly bound.

In figure 1 anaerobic carbon dioxide production (as percentage of activity

retained) is plotted against the logarithm of the vapor pressure. The original values for samples B and C were, respectively, 391 and 354 microliters of carbon dioxide per mg of oven-dry yeast per hour. About 50 per cent of the activity was retained at all lower levels of water retention. At 26 mm vapor pressure, however, trouble was experienced from decomposition. Sample B, which underwent some decomposition, lost much activity, but sample C retained almost all of its original activity. This may be attributed to autolysis of the yeast because of the slow drying at this high vapor pressure.

CONCLUSIONS

Yeast dried at low vapor pressures (0.0016 to 0.1 mm mercury) retained 0.03 gram of water per gram of yeast. No additional water was removed by drying over concentrated sulfuric acid.

The activity of the yeast as measured by the amount of anaerobic carbon dioxide production fell about half its original value when the yeast was dried at 10 mm vapor pressure, but drying at lower vapor pressures caused no further loss of activity.

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- BLAKE, F. C., AND GREENEWALT, C. H. 1928 Vapor pressure, normal boiling points and latent heat of vaporization for aqueous solutions of sulfuric acid. *International Critical Tables*, 3, 302-303. McGraw Hill Book Co., New York.

NOTES

PARACOLON TYPE 10 FROM CAPTIVE RATTLESNAKES

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During the spring of 1942 a paracolon type was isolated by the authors from a captive rattlesnake at the San Diego Zoo. This culture (Pc27) was studied by Edwards, Cherry, and Bruner (*J. Infectious Diseases*, **73**, 229) and designated by them as "type 10." The antigenic formula is XVIII z_4 x_2 x_7 . Since that time this type has been found to be the cause of considerable mortality in turkey poults in the same area (Hinshaw and McNeil: *J. Bact.*, **51**, 281). The present paper reports further isolations from the same cage (A-31) of rattlesnakes from which the original one was made.

Acc. 144-1. This male rattler (*Crotalus viridis-oreganus*) died on June 1, 1942. The only symptom of previous illness was that it had disgorged the rat fed to it on February 1, 1942. However, at death it was fat and in a good state of preservation. On necropsy the organs did not show the usual enzymic decomposition noted in dead reptiles. This indicates that death was the result of an acute disease. The only lesions were numerous small, necrotic areas in the liver. From these lesions a type 10 paracolon (Pc27) was isolated in pure culture; other organs were not cultured. This snake had been in the cage since its capture in June, 1937, in Lower California.

Acc. 235-2. On September 7, 1943, an emaciated sidewinder (*Crotalus cerastes*) of unknown history, was killed. With the exception of an enlarged gall bladder all organs appeared normal. From the liver of this snake a paracolon type was isolated which has the flagellar antigens of type 10, but a new somatic antigen.

Acc. 235-3. On September 7, 1943, a type 10 paracolon was recovered from a fecal sample from this cage.

Acc. 235-9. On September 28, 1943, a female prairie rattler (*Crotalus viridis*) died. It had been in this cage since June 6, 1943, and had not eaten since that time, although it undoubtedly drank water during those months. Type 10 was recovered from the kidney, lungs, and uterus.

Acc. 235-17. On October 17, 1943, a young female rattlesnake (*C. viridis-oreganus*) died. It had been in cage A-31 since June 23, 1943. The bile was thick and "tarry"; other organs appeared normal. Type 10 was isolated from the liver, ovary, lungs, heart blood, kidney, and intestine.

Acc. 235-64. On January 19, 1944, a male sidewinder (*Crotalus cerastes*), of

¹ We wish to express our appreciation for the cooperation of the following people: Mr. C. B. Perkins, Curator of Reptiles, San Diego Zoo; Mr. T. J. Taylor, University of California, Davis; and Dr. P. R. Edwards, University of Kentucky, Lexington.

unknown history, died, and from its lungs a paracolon type was isolated but was lost before it could be identified antigenically.

Acc. 270-38. On September 13, 1944, a sidewinder (*C. cerastes*) died. It was very poor, the rectum was congested, and the liver was studded with minute abscesses. It had been in this cage since September 1, 1943, and had not eaten for almost a year. Type 10 was recovered from the heart blood, lungs, liver, kidneys, and intestine.

270-46. On November 8, 1944, a male sidewinder rattlesnake (*C. cerastes*) died. It had been in this cage only a short time. It was poor, the heart was flabby, and there were numerous cysts in the abdominal cavity. No paracolon types were recovered.

270-53. In April, 1945, two feces samples were taken from this cage, which by then had only two survivors in it. Paracolon types were not recovered; *Pseudomonas* strains predominated in both specimens.

A total of 15 snakes have been autopsied for the San Diego Zoo since 1942. Seven of the 15 were rattlesnakes from this cage (A-31). Paracolon type 10 was isolated from at least 4 of these. A paracolon strain isolated from a fifth snake was not identified antigenically; biochemically it was similar to the others. Feces from this cage, cultured in 1943, also yielded type 10 paracolon.

There are several facts which show the relationship of the isolations from these snakes to paracolon type 10 infection of turkeys. This cage was always kept as an exhibit of snakes caught in San Diego County or adjoining territory. This is the only cage which suffered epidemic losses, and the only one from which this paracolon type was recovered. The losses caused by this type in turkey poults referred to above were also confined to this area, and rattlesnakes were present on at least two of the turkey ranches from which this type was isolated.

AN OBSERVATION OF APPARENT SUBSTITUTION OF PANTOTHENATE BY THIAMINE AND CHOLINE¹

CARL LAMANNA AND CHARLES LEWIS²

Camp Detrick, Frederick, Maryland

Received for publication February 1, 1946

In a study of the growth factors required by the Hall strain of *Clostridium botulinum*, type A, an interesting apparent substitution by thiamine and choline for calcium pantothenate has been observed. The experiments utilized a minimal inoculum. Ten ml of medium were inoculated with 0.1 ml of a 1:50 dilution of washed cells from 18- to 24-hour-old cultures grown in agar-free Brewer's thioglycollate medium. The inoculum culture, before dilution, was washed three or four times by centrifuging from original culture volume in a sodium

¹ Work conducted at Camp Detrick, Frederick, Md., from Oct., 1944, to Dec., 1944.

² Ens., USNR.

chloride, phosphate buffer solution (0.8 per cent NaCl plus one per cent of an equimolar mixture of $\frac{1}{15}M$ K_2HPO_4 and KH_2PO_4). For each day's work fresh inoculum was prepared. Inasmuch as an acid-hydrolyzed vitamin-free casein basal medium did not support growth, the following growth factors were tested as supplements (quantities given are per 10 ml of medium): biotin, 0.005 μg ; choline, 25 μg ; thiamine, 0.2 to 4 μg ; calcium pantothenate, 5 μg ; pyridoxine, 10 μg ; nicotin, 5 μg ; riboflavin, 1 μg ; inositol, 200 μg ; *p*-aminobenzoic acid, 5 μg ; folic acid concentrate, 0.15 μg of potency 5,000 (Univ. Texas Pub., **4137**, 37).

All of the individual growth factors tested were added to the basal medium and then one factor was removed at a time. Irrespective of the component missing, growth occurred except in the case of the biotin-deficient medium. These results

TABLE 1

*Least number of components of vitamin mixtures supporting growth of Clostridium botulinum type A (Hall strain) in an acid-hydrolyzed casein medium**

MEDIUM	BIOTIN	CA-PANTOTHENATE	THIAMINE	CHOLINE	FOLIC ACID CONCENTRATE
1	P†	P			
2	P	P		P	
3	P	P	P	P	
4	P	P			P
5	P		P	P	
6	P	P	P	P	P

* Basal medium consisted of the following ingredients per ten ml of medium: acid-hydrolyzed vitamin-free casein (10 per cent solution), 0.5 ml; glucose (0.5M), 0.5 ml; tryptophane (51 mg per cent), 0.1 ml; salts B, 0.01 ml; phosphate buffer (0.1M, pH 7.2), 0.1 ml; sodium thioglycollate (5 per cent solution), 0.1 ml.

† P = vitamin present.

were repeatable. It is evident that biotin cannot be synthesized by the organism. However, biotin alone, when added to the basal medium, did not support growth. Various combinations of the vitamins were tried with growth occurring in those mixtures listed in table 1. The same results were obtained with vitamin mixtures when the basal medium was further supplemented with adenine, guanine, *iso*-cytosine, and uracil, which alone in combination with the basal medium did not support growth.

From the results obtained, it was concluded that biotin was essential but not sufficient in itself. Biotin and calcium pantothenate in combination supported slight growth. The addition of thiamine, choline, pyridoxine, and folic acid increased the amount of growth. Thiamine, choline, and biotin supported growth without the presence of pantothenate.

A possible explanation of the results obtained is that the capacity of the organism to store pantothenic acid, thiamine, and choline is minimal so that when these materials are not provided by the external environment growth cannot be initiated. An external supply provides an initial source that can then be supplemented by synthesis during cell growth.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

KENTUCKY BRANCH

UNITED STATES PUBLIC HEALTH SERVICE HOSPITAL, LEXINGTON, KENTUCKY,
NOVEMBER 10, 1945

SOME OBSERVATIONS ON THE CONTROL OF FOWL TYPHOID INFECTION WITH SULFA DRUGS. *D. Frank Holtman and Gladys Fisher*, Department of Bacteriology, The University of Tennessee, Knoxville, Tenn.

An epidemic of fowl typhoid appearing in a flock of 400 week-old battery-raised chicks caused 20 per cent mortality within 3 days. At this time the flock was divided into two groups. Group I received the usual care, consisting of removal of sick chicks, cleaning and disinfection of the battery with cresol solution. Group II received no such care but instead was given 0.1 per cent sodium sulfathiazole in drinking water for a period of one week.

At the end of the week, 80 per cent of the chicks in Group I had died. Losses in Group II had been reduced to 4 per cent, with no mortality during the last 3 days of the week.

The chicks in Group II were taken off sulfa drug treatment and within 5 days the infection reappeared. Drinking water containing 0.1 per cent sodium sulfathiazole was again supplied for one week and the epidemic was checked with a 2 per cent loss. After a second week without treatment another outbreak of the infection occurred, and the chicks were returned to still another week of sulfa drug treatment. At the same time, the battery was thoroughly cleaned and disinfected, and no further losses occurred after discontinuance of the treatment.

STUDIES ON COMPLEMENT FIXATION WITH MYCOLOGICAL MATERIAL. *Margaret Hotchkiss*, Department of Bacteriology, University of Kentucky, Lexington, Ky.

A fungus obtained from material aspirated from the bronchi of a patient and identified as a *Sporotrichum* sp. was used to prepare a vaccine. After prolonged im-

munization of rabbits, serum was produced which could be used in complement-fixation tests. Paper-filtered cultures of fungi were used as antigens after the anticomplementary activity had been titrated.

By the use of a constant amount of antigen and graded dilutions of serum, complement fixation was obtained with the homologous organism, and slight fixation with unrelated fungi. *Geotrichum* sp., *Penicillium notatum*, and *Trichophyton gypsum* have been tested. On the other hand, a strain of *Sporotrichum schenckii* gave complete fixation with 0.005 ml of antiserum, but partial fixation with 0.001 ml of antiserum, although the homologous organism gave complete fixation.

SUBMERGED CULTURE OF MOLDS FOR AMYLASE PRODUCTION. *Floyd Stewart, R. E. Scaif, and W. H. Stark*, Joseph E. Seagram and Sons, Inc., Louisville, Ky.

Mold amylases as saccharifying agents have been widely employed in the Orient, as in the production of Japanese sake, in Europe in the amylo process, and to a limited extent in the United States. The disadvantage in the use of mold amylases, as generally produced and employed, lies in the difficulty of producing the amylases in quantity, free of bacterial contamination, by surface culture methods.

Studies on submerged culture of molds have shown that such cultures could be grown free of bacteria, making them especially adaptable for use in the conversion of grain mashes in a continuous fermentation process. Investigations to determine the optimum deep culture growth conditions for maximum amylase production, with the selection of the most suitable strains of mold for amylase production, were conducted. Twenty-one strains of molds of

the genera *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* were tested.

The amylolytic and saccharogenic values of the amylases have been increased from 300 and 600, respectively, to 2,400 by the selection of molds and by determining the optimum conditions of medium, pH, and aeration for amylase production.

DISINFECTION OF DISTILLERS' BARLEY

MALT BY CHEMICAL AGENTS. *Arthur A. Anderson, E. W. Ruf, and W. H. Stark, Joseph E. Seagram and Sons, Inc., Louisville, Ky.*

CONTINUOUS ALCOHOLIC FERMENTATION OF ACID-HYDROLYZED GRAIN MASHES. *E. W. Ruf, W. H. Stark, and Leroy A. Smith, Joseph E. Seagram and Sons, Inc., Louisville, Ky.*

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND EIGHTY-THIRD MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., NOVEMBER 27, 1945

A CHICK EMBRYO TECHNIQUE FOR INTRAVENOUS AND CHEMOTHERAPEUTIC STUDIES. *Henry F. Lee, Abram B. Stavitsky, and Margaret P. Lee, University of Pennsylvania, School of Medicine, Department of Pediatrics and The Children's Hospital of Philadelphia, Philadelphia, Pa.*

This technique, which was devised for the pilot testing of chemotherapeutic agents against bacterial infections of the chick embryo, is briefly as follows: The shell over the air sac is removed, and the shell membrane is stripped from the underlying chorio-allantoic membrane. The embryonic sacs are then clearly seen, and selective or combined intravenous, intrayolk, intra-amniotic or intra-allantoic injections or aspirations may be made accurately. Five one-hundredths ml of fluid may be inoculated into very small allantoic venules, or blood may be aspirated using a very sharp 27-gauge needle and a 0.25-ml tuberculin syringe. The open end of the egg is sealed with two layers of Scotch tape through which the embryo is visible, permitting ready determination of its death without candling. During incubation substances may be applied to the membrane by passing a fine needle through the tape or may be aspirated from the embryo by removing the tape.

The results of intravenous injection of

chick embryos with human tubercle bacilli will be published separately. Sharp end points in mortality with varying concentrations of promin, sulfathiazole, and other drugs and consistent body fluid levels of promin and sulfathiazole were obtained in embryos by this technique.

THE ANTIGENS OF EPIDEMIC TYPHUS VACCINE. I. THE DISTRIBUTION AND IMMUNOLOGICAL PROPERTIES. *Leslie A. Chambers, Seymour S. Cohen, and Jean R. Clawson, Johnson Foundation for Medical Physics and the Department of Pediatrics, School of Medicine, University of Pennsylvania, and The Children's Hospital of Philadelphia, Philadelphia, Pa.*

This material will be published in the Proceedings of the First Inter-American Conference on Typhus, Mexico City, October 7th to 15th, 1945.

THE ANTIGENS OF EPIDEMIC TYPHUS VACCINE. II. THE CHEMICAL COMPOSITION OF RICKETTSIA PROWAZEKI. *Seymour S. Cohen, Leslie A. Chambers, and Jean R. Clawson, Johnson Foundation for Medical Physics and the Department of Pediatrics, School of Medicine, University of Pennsylvania, and The Children's Hospital of Philadelphia, Philadelphia, Pa.*

OHIO BRANCH

OHIO STATE UNIVERSITY, COLUMBUS, OHIO, DECEMBER 1, 1945

OBSERVATIONS ON THE CHEMOTHERAPEUTIC PROPERTIES OF THE SULFANILANILIDES. *Clara L. Sesler and L. H. Schmidt. In-*

stitute of Medical Research, Christ Hospital, Cincinnati, Ohio.
Thirty-two sulfanilamide derivatives

were examined for *in vivo* and *in vitro* activities against pneumococci and Friedlander's bacilli and for the effects of *p*-aminobenzoic acid on these *in vitro* activities.

The *in vivo* activities of these drugs were not promising. None of the compounds were so active as sulfanilamide against infections with either pneumococci or Friedlander's bacilli.

In vitro many of the sulfanililides showed high activities against the pneumococci, some 15 of the compounds having approximately the same activity as sulfathiazole. The position and nature of the substituent were important factors in determining this activity, the most effective points of substitution being the 3' and 3', 5' positions and the most effective substituents being halogen, cyano-, or nitro-groupings.

In vitro activities against Friedlander's bacilli were uniformly low. None of the sulfanililides were significantly more effective than sulfanilamide, the least active of the common sulfonamides; most of the compounds were less active.

Wherever tests were possible, the activities of the sulfanililides against Friedlander's bacilli were uniformly blocked by *p*-aminobenzoic acid. However, the activities of many of these drugs against the pneumococci were either not at all affected by *p*-aminobenzoic acid or were only partially antagonized.

ACTION OF PENICILLIN ON *Staphylococcus* IN VITRO. Harriet Marsh and R. F. Parker.

When a 4-hour culture of *Staphylococcus* is exposed to an adequate concentration of penicillin, the number of viable bacteria in the culture decreases progressively and regularly during the first 2 hours of exposure. The *Staphylococcus* used in these experiments was inhibited by 0.06 U of penicillin per ml. Addition of 1.0 U per ml to a 4-hour culture gave the effect described above.

The effect of short exposure to *Staphylococcus* to penicillin was tested as follows: To a 4-hour culture, penicillin was added in an amount sufficient to yield the desired concentration. The culture was returned to the incubator at 37 C. After a predetermined interval the culture was removed,

an appropriate amount transferred to a centrifuge tube, and centrifuged for five minutes. The supernatant was pipetted off, replaced with warm broth, and the culture recentrifuged. The second supernatant was removed, replaced with an amount of warm broth equal to the original volume, and returned to the incubator. When the culture was exposed to 1.0 U of penicillin per ml for 5 minutes, or 0.06 U for 30 minutes, there was no appreciable killing of bacteria. However, on reincubation of the culture, growth was resumed only after a period of 3 hours had elapsed. It then proceeded normally.

THE EFFECT OF STARVATION ON THE DEVELOPMENT OF IMMUNITY IN MICE AFTER VACCINATION WITH WESTERN EQUINE ENCEPHALOMYELITIS VIRUS. Isaac Ruchman, The Children's Hospital Research Foundation and the Department of Bacteriology, University of Cincinnati School of Medicine, Cincinnati, Ohio.

The influence of starvation on the development of immunity in mice after vaccination with a formalinized Western equine encephalomyelitis mouse brain virus was studied. Immunity was determined by the development of cerebral resistance and of neutralizing antibodies. The latter was quantitatively determined by the method of serum dilution as well as the usual method employing undiluted serum. Mice were tested 2 weeks after the initial dose of vaccine.

The well-nourished vaccinated mice resisted between 100 and 200 times more virus intracerebrally than did the underfed animals. The test for neutralizing antibodies likewise showed a significant difference between undernourished and well-nourished mice, the well-nourished animals neutralizing between 10 and 100 times as much virus as did the starved mice. The serum of the adequately nourished animals could be diluted between 3 and 8 times more than could the serum obtained from the undernourished mice and still neutralize the effects of a constant amount of virus (50 LD₅₀ doses). Prolonging the period of starvation for an additional two weeks did not accentuate the depressed immunity response.

MUTATION OF *ASPERGILLUS NIGER* VAN TIEGHEM BY MEANS OF SOFT X-RAYS. Violet M. Diller, Alfred A. Tytell, and H. Kersten, Departments of Physics and Biological Chemistry, University of Cincinnati, Cincinnati, Ohio.

A mutant of *Aspergillus niger* (Van Tieghem) was produced by irradiation with soft X-rays. The mutant differed from the original culture in appearance and in growth characteristics. Experiments were conducted to show differences in metabolism which might exist between the two. Flasks containing a modified Czapek's liquid medium were inoculated with both irradiated and nonirradiated *A. niger*. At regular intervals quantitative determinations were made of the amount of citric acid produced, the amount of sugar utilized, and the weight of the resulting mat.

That the metabolism of the two forms differs and that the mutant is more efficient in the production of citric acid are evidenced by the fact that at the peak of production the mutant produced 50 per cent more citric acid, with the consumption of less sugar, than did the original organism. The mutant also grew more slowly and produced less mat. The ratio of citric acid to weight of mat was larger for the irradiated culture; also, the ratio of mat weight to sugar utilized was greater for the mutant.

RAPID ASSAY METHOD BASED ON NITRATE REDUCTION FOR ANTIBIOTICS ACTIVE AGAINST *ESCHERICHIA COLI*. F. A. Nielsen and Milton J. Foter, Department of Bacteriology, Research Laboratories of The Wm. S. Merrell Company, Cincinnati, Ohio.

An assay method based upon the ability of *Escherichia coli* to reduce nitrate to nitrite has been developed. The test is rapid, requiring 3 hours' incubation, and its accuracy is considered to be equal to that of the cup-plate assay for penicillin.

THE USE OF STREPTOMYCIN IN THE PURIFICATION OF CULTURES OF *TRICHOMONAS VAGINALIS*. Robert A. Quisno and Milton J. Foter, Department of Bacteriology, Research Laboratories of The Wm. S. Merrell Company, Cincinnati, Ohio.

Following the isolation of a bacteria-free culture of *Trichomonas vaginalis* by Trussell in 1939, several investigators reported the use of penicillin in obtaining pure cultures of this parasite. Since gram-negative species are, for the most part, resistant to the action of penicillin, this purification technique is limited in use. The chance contamination of a pure culture of *Trichomonas vaginalis* with *Escherichia coli* and *Staphylococcus aureus* and the subsequent attempts to repurify it with 10 units per ml of penicillin indicated that this procedure had a limited application since the gram-negative species could not be eliminated from the mixed culture. Cultures of *Trichomonas vaginalis* contaminated with gram-positive and gram-negative bacterial species were purified by the addition of 25 units per ml of streptomycin after 10 hours' exposure at 37 C. Subcultures were made in a cysteine, peptone, liver infusion, maltose medium. The exposure time for purification was reduced by several hours by employing a higher unitage of streptomycin. Crude streptomycin preparations and crude filtrates were used with success in purifying cultures of this parasite.

Rapid purification of cultures of *Trichomonas vaginalis* was obtained with streptomycin. Its application in the examination of vaginal discharges in acute or subacute trichomonas vaginitis, as reported by Johnson (Science, 102, 126), has added advantages.

ON THE EFFICIENCY OF COTTON PLUGS IN MAINTAINING STERILITY. Orton K. Stark, Miami University, Oxford, Ohio.

Some cotton-plugged test tubes containing applicator sticks with swabs were found to be sterile after 21 years on an office shelf. This indicates the effectiveness of cotton plugs in preventing contamination.

THE RECIPROCAL SENSITIVITIES OF *STAPHYLOCOCCUS AUREUS* TO PENICILLIN, STREPTOMYCIN, AND STREPTOTHRICIN AFTER THE DEVELOPMENT OF FASTNESS TO EACH DRUG SEPARATELY. Marguerite Sullivan, Grant L. Stahly, Wm. G. Myers, and Jorgen M. Birkeland, The Department of Bacteriology and The Department of

Medical Research, The Ohio State University, Columbus, Ohio.

Cincinnati, College of Medicine, Cincinnati, Ohio.

ACTIVE IMMUNIZATION OF CHILDREN WITH DYSENTERY VACCINES. *Merlin L. Cooper, Jack Tepper, and Helen M. Keller*, Children's Hospital Research Foundation, Department of Pediatrics, University of

A QUANTITATIVE METHOD FOR ASSAY OF BACTERIAL FLORA OF WOUNDS AND ITS USE IN EVALUATION OF CHEMOTHERAPY. *Morris Schaeffer*, City Hospital, Cleveland, Ohio.

NEW YORK CITY BRANCH

THIRTY-FIFTH MEETING, GEORGE WASHINGTON HOTEL, NEW YORK,
N. Y., DECEMBER 27, 1945

VITAMIN REQUIREMENTS OF THE PHOTOSYNTHETIC PURPLE BACTERIA. *S. H. Hutner*, Haskins Laboratories, New York.

Investigation of the vitamin requirements of 124 isolates of purple bacteria kindly supplied by Prof. C. B. van Niel revealed that each of the 5 species studied had a different requirement. The vitamin requirements were remarkably uniform within each species.

Of 17 isolates previously identified as *Rhodospirillum rubrum*, 15 required biotin; 2 did not grow in the synthetic medium. All 34 isolates of *Rhodopseudomonas palustris* required *p*-aminobenzoic acid. Fourteen of the 15 isolates of *Rhodopseudomonas capsulatus* required thiamine; one did not grow. All 20 isolates of *Rhodopseudomonas gelatinosa* required biotin + thiamine; the 17 isolates of *Rhodopseudomonas spheroides* required biotin + thiamine + nicotinic acid. Twenty-one additional miscellaneous unclassified isolates were assigned to the proper species on the basis of vitamin requirements.

In later work the sole organic constituents of the medium were synthetic malic acid and Na-acetate. For good growth with these relatively pure compounds a complex assortment of trace elements had to be supplied. The very favorable effect previously observed of natural amino acids and protein hydrolyzates was found to depend largely on their content of essential trace elements; otherwise the utilizable amino acids presented no advantage over the better N-free C and H sources.

STUDIES ON BACTERIAL MUTABILITY. THE TIME OF APPEARANCE OF THE MUTANT. *Stephen Zamenhof*, New York.

APPLICATION OF NEW BACTERIOLOGIC METHODS TO THE STUDY AND CONTROL OF STAPHYLOCOCCUS FOOD POISONING OUTBREAKS. *George H. Chapman and Emil Domingo*, Clinical Research Laboratory, New York.

Atypical reactions of suspected food poisoning staphylococci may have been caused by the use of unsuitable bacteriological technique. Using the cultural methods described by the senior author (see below) in studying local outbreaks, all incriminated strains of staphylococci produced orange pigment, clotted blood, fermented mannitol, and produced considerable zoning of the Stone type *when tests were made at the time of isolation*. The direct coagulase test was positive in 2 hours in food containing 231,000,000, in 7 hours in food containing 750,000, staphylococci per gram. The complete bacteriological examination took 48 hours. There was no interference from other bacteria. Strains with similar reactions and with similar degrees of Stone zoning were isolated from the nasal cavities of suspected vectors. After an outbreak, handlers were instructed in hygiene and subsequent food was tested to be sure that the measures were effective. Cultures were also made on bacto violet red bile agar for coliforms and on Chapman's tellurite streptococcus medium to check on personal hygiene.

A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF TYROSINE IN PROTEINS AND FOODS. *Marion Gunness, Irla M. Dwyer, and Jacob L. Stokes*, Merck and Co., Rahway, N. J.

The previously developed basic microbiological assay method for the determination of the ten essential amino acids in pro-

teins and foods has been extended to include the assay of tyrosine. The method is based upon the quantitative response of *Lactobacillus delbrückii* LD5 to increments of 0 to 100 μ g of tyrosine as measured by titration of the lactic acid produced during growth with standard alkali. The reliability of the method is supported by the good agreement of values for different amounts of sample assayed, reproducibility of values on repeated assay, and the quantitative recovery, within the usual microbiological variation of 10 per cent of tyrosine added to proteins prior to hydrolysis. As much as 50 per cent of the tyrosine in foods with a high carbohydrate content may be lost due to humin formation if hydrolysis with acid is employed. Such losses do not occur if hydrolysis is conducted with alkali and, therefore, the latter is routinely applied to all natural materials. Either acid or alkali can be used to hydrolyze purified proteins. The microbiological tyrosine values for the 15 natural materials and 7 purified proteins assayed are in fair agreement, in most instances, with chemical values on similar substances cited in the literature.

THE LACK OF PRESERVATIVE ACTION OF SURFACE-ACTIVE CATIONIC GERMICIDES IN MILK. *Adrien S. DuBois and Diana D. Dibblee*, Onyx Oil and Chemical Co., Jersey City, N. J.

The bacterial count of raw milk was not affected by the presence of 1:500 to 1:25,000 dilutions of alkyldimethylbenzyl-ammonium chloride, upon incubation at either 10, 20, or 37 F. However, 1:500 and 1:1,000 dilutions of the germicide caused an immediate, appreciable reduction in the initial count of the milk. Less acid was produced in the treated milk during incubation. This was especially noticeable with the higher concentrations of the germicide.

The lower acidity in the treated milk is assumed to be due to the inhibition of the gram-positive acid-forming organisms by the alkyldimethylbenzyl-ammonium chloride. The gram-negative rods are not inhibited and this accounts for the high bacterial counts. Qualitative evidence for this was obtained by identification of the organisms growing under the various conditions of test.

Concentrations of surface-active cationic germicides varying from 1:500 to 1:20,000 can easily be determined in milk by titration with duponol PC in the presence of bromophenol blue.

FUNGUS DETERIORATION TESTING. A CHEMIST'S CONTRIBUTION. *Jack Marshall*, Advance Solvents and Chemical Corp., New York.

Several recommendations are made: (1) The use of an agar medium consisting chiefly of mineral nutrients, some sugar, but no peptone. The use of both primary and secondary potassium phosphates automatically creates the desired solution having a pH of about 6.8 without adjustment with acid or alkali. The medium contains in 1 liter of water: 1 g ammonium nitrate, 0.75 g dipotassium hydrogen phosphate, 0.75 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, 10 g brown sugar, and 10 g agar. Reasons were given for the importance of this combination of ingredients, and comparison was made with other test media. (2) Inoculation of the medium before pouring into petri dishes, allowing a considerable saving of time and material, and producing a smooth fungus mat; this eliminates the pipette entirely. (3) Modified pipettes for individual inoculation. (4) Suggestions for incubator construction, and details for inverting petri dishes during incubation. The development and reasons for these recommended changes were discussed.

OBSERVATIONS ON ANTIMICROBIAL ACTION OF 2,3-DICHLORO-1,4-NAPHTHOQUINONE, AND ITS REVERSAL BY VITAMINS K. *D.W. Wooley*, Rockefeller Institute for Medical Research, New York.

2,3-Dichloro-1,4-naphthoquinone, an antifungal agent now in practical use, has been recognized as an analog of vitamin K. This substance has been found to be exceedingly toxic to yeasts and moderately harmful to the growth of bacteria. Its effect on yeast was reversed competitively by vitamins K over a limited range of concentration. Although the effect on the growth of bacteria was not influenced by vitamin K in the form of 2-methyl-naphthoquinone, it is probable that this was due to

the toxicity of this form of the vitamin for these species.

FURTHER STUDIES ON THE PABA-RESISTANT SULFONAMIDES: THE SULFANILYLANILIDES.

G. R. Goetchi and *C. A. Lawrence*, Winthrop Chemical Co., Rensselaer, N.Y.

Further studies have been made to augment the preliminary work on benzene-sulfonic acid derivatives (*J. Bact.*, *49*, 575). 3',5-Dibromosulfanililide, which is unaffected by the presence of PABA, was found to be highly effective against such pathogens as pneumococci, meningococci, gonococci, hemolytic streptococci, staphylococci, Ducrey's bacillus, strains of *Brucella* and *Clostridium*, *Vibrio cholerae*, *Listerella monocytogenes*, and *Actinomyces bovis*.

A MICRO-PLATE METHOD FOR PENICILLIN ASSAY. *Edith R. Jackson*, American Cyanamid Co., Stamford, Conn.

A method is presented for the microbiological assay of penicillin wherein a standard solution and a sample of blood or other body fluid are titrated simultaneously for inhibition end points against group A *Streptococcus*, strain C-203. The test consists of a series of dilutions of the sample in blood plus streptococcus-seeded agar, totaling 0.5 ml, in flat-bottom shell vials of 18-mm diameter. The micro-plate test requires no subculture, can be performed without strict sterility, and appears to be more sensitive than other tests now frequently used.

A METHOD FOR RESTORING AND MAINTAINING THE PHENOL RESISTANCE OF CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS. *Thomas C. Grubb* and *Marguerite A. Edwards*, Vick Chemical Co., New York.

TOXICITY OF GERMICIDES. *Louis C. Barail*, United States Testing Co., Hoboken, N. J.

The purpose of the tests was to find a germicide which will render light fabrics, to be worn next to the skin, sterile and germicidal after a great number of washings. Over 250 germicides were tested. All compounds were previously studied for toxicity,

presence of skin irritants, and cutaneous sensitizers.

First, toxicity tests and minimum lethal dose determinations were made on the compounds. Then, intradermal animal injections and patch tests on human skin were conducted for the presence of skin irritants and cutaneous sensitizers. The patch test method used was that recommended by Drs. Louis Schwartz and Samuel Peck of the U. S. Public Health Service. It consists in applying a patch on a minimum of 200 individuals for five days, observing the skin after removal and the following 2 days, and applying a similar patch for 48 hours 10 days after removal of the first one.

Out of 250 compounds, 7 only were acceptable because of their low toxicity, and among these 5 were eliminated as skin irritants and cutaneous sensitizers. One compound which is not a skin irritant was found to be a cutaneous sensitizer. Only one is neither a skin irritant nor a cutaneous sensitizer, even at concentrations higher than the normal concentrations of use.

The patch test method indicated that the only satisfactory one for lack of toxicity is a long chain mercurial compound (lactoxyphenylmercuric ammonium lactate) which renders light fabrics germicidal after as many as 40 washings.

INFLUENCE OF SYNTHETIC ESTROGEN UPON EXPERIMENTAL INFECTIONS IN ANIMALS.

E. W. Blanchard, *B. Heinemann*, and *R. B. Stebbins*, Schieffelin and Co., New York.

The purpose of this study was to investigate the influence of chemically pure synthetic compounds of high estrogenic potency on experimental infections in laboratory animals. Rats and mice were treated with two highly active racemates of benzenestrol, a synthetic estrogen, for 10 days prior to infection. The mice were then infected intraperitoneally with a virulent culture of *Diplococcus pneumoniae*, type I, and the rats were infected intraperitoneally with a virulent strain of *Treponema equiperdum*. The courses of the infections were compared with similar infections in untreated controls.

Although the estrogens gave no marked protection against either infection in mice or rats, the degree of prolongation of survival following infection appears to be significant. Female mice, infected with *D. pneumoniae*, lived 40 per cent longer than the controls, and male rats, infected with *T. equiperdum*, survived 60 per cent longer than the controls. This increase is sufficiently significant to warrant further investigation.

HEAT-LABILE SOMATIC ANTIGENS OF SHIGELLA. *A. J. Weil and M. M. Binder*, Lederle Laboratories, Pearl River, N. Y.

Two recently described non-mannitol-fermenting strains of *Shigella*, *S. sp. Sachs Q454* (from India) and *S. sp. Wakefield* (described by Berger in England) are distinct in their cultural behavior and in their heat-stable, type-characteristic antigen. However, they have in common an antigen which is destroyed by boiling. Similar observations have been made by Braun on *Flexner bacilli* and by Kauffmann on *colon bacilli*. Details will be reported in a forthcoming paper in the *Journal of Immunology* (1946).

THE WHITE LABORATORY RAT: A HOST FOR HUMAN THROAT INCLUSION BODIES. *Jean Broadhurst*, Professor Emeritus, Teachers College, Columbia University, New York.

The throat inclusion bodies previously reported in man by the author and several associates have been found in scrapings or teased preparations from the tongue, inner cheek, or genital area of slightly over one-third of 50 freshly killed white rats. Although inclusion bodies were observed in a few of the 20 rats on normal diets, the incidence was three times as high in the 30 rats on diets deficient in protein, calcium, or vitamins A, B₁, D, or G. The incidence was four times as high in rats deficient in A or D; and the inclusions were much more numerous in four of the vitamin-A-deficient rats than in any other rats.

The number of rats examined is too small to warrant any conclusion regarding the relation of the diet to the incidence of these inclusion bodies. Of greater interest is the fact that the five laboratory workers in one

laboratory handling most of the positive rats were all strongly positive for these inclusion bodies, suggesting a rat-man relationship for this virus not heretofore recognized.

THE SIGNIFICANCE OF TYPE-SPECIFIC MENINGOCOCCIC AGGLUTININS IN HUMAN SERUMS. *Carolyn R. Falk and Emanuel Appelbaum*, Bureau of Laboratories, New York City Department of Health.

The agglutination test using patient's serum and antigens representative of the prevailing types of meningococci of well-established antigenicity and specificity may be used to confirm a clinical diagnosis of meningococcic infection in those cases in which other laboratory methods have failed. In cases in which the clinical diagnosis is questionable the possibility of gonococcic infection or exposure to active meningococcic infection must not be overlooked. A typical rise in titer when successive samples are tested during the various stages of the disease substantiates the laboratory diagnosis by the agglutination method.

EXPERIMENTAL INFLAMMATIONS OF THE HEART. *Ward J. MacNeal, Anne Blevins, and Alice E. Slavkin*, New York Post-Graduate Medical School.

INACTIVATION OF VIRUSES. *Katherine Wardell, Helen Scanlon, and Ward J. MacNeal*, New York Post-Graduate Medical School.

CONJOINED ACTION OF ANTIBIOTICS. *Louise Filak, Anne Blevins, and Ward J. MacNeal*, New York Post-Graduate Medical School.

ANTIGENICITY STUDIES OF GAS GANGRENE TOXOIDS. *A. M. Webb, F. L. Clapp, I. S. Danielson, and C. H. Parsons*, Lederle Laboratories, Pearl River, N. Y.

ANTITOXIN TITERS FOLLOWING IMMUNIZATION WITH PROTAMINE-PRECIPIATED DIPHTHERIA TOXOID. *Victor Ross*, Columbia University College of Physicians and Surgeons, New York, *Frances L. Clapp and Bertha W. Schimpf*, Lederle Laboratories, Pearl River, N. Y.

The preparation of protamine-precipi-

tated toxoid has been described previously by one of us. One injection of 1 ml (20 to 25 Lf) converted 96 per cent of 554 children from a positive to negative Schick state.

The present study revealed that 78 per cent of 46 children produced between $\frac{1}{10}$ and $\frac{1}{2}$ of a unit of antitoxin per ml of serum, whereas 26 per cent had $\frac{1}{10}$ of a unit or more between 2½ and 18 months after one injection (20 to 25 Lf). Two children had $\frac{1}{10,000}$ unit. Between 11 and 18 months after one injection 30 per cent had $\frac{1}{10}$ unit or more. Seventy-nine per cent of 34 children produced $\frac{1}{10}$ of a unit or more, and 60 per cent $\frac{1}{2}$ unit or more, 2½ to 3 months after the second of 2 injections, whereas 90 per cent of 60 children similarly tested produced $\frac{1}{10}$ unit or more, and 58 per cent $\frac{1}{2}$ of a unit or more, at 9 to 15 months. Comparison of our data with those of Volk and Bunney shows that, both with one and two doses, more children produce $\frac{1}{10}$ of a unit or more when protamine toxoid is used than when alum toxoid is employed. Also there is less tendency for the titer to fall after 12 months. Our children were from an urban population and 80 per cent were not over one year old, whereas theirs were from rural areas and $\frac{1}{3}$ were 6 to 10 years old. To what extent these facts may have contributed to the better results is as yet unknown. The almost complete absence of bacillary protein from the new preparation, its low nitrogen content, and the lesser reactions observed following simultaneous intradermal injections of protamine, alum, and "fluid" toxoid recommend its use.

OBSERVATIONS ON EARLY SKIN REACTIONS TO TUBERCULIN. Janet McCarter, Columbia University College of Physicians and Surgeons, New York.

A skin reaction to tuberculin which appears within 6 hours and may or may not last for 24 hours or more has been described by McCarter and Watson. The speed with which a reaction appears and reaches its maximum has now been shown to depend upon variation in the host; the reaction appears regardless of whether heated or unheated protein has been used. The proteins employed had been prepared from both heated and unheated tubercle bacillus culture filtrates by ammonium sulfate frac-

tionation. Most of the experiments were done with a native protein homogeneous ultracentrifugally (determined by Dr. Ellen Bevilacqua) and serologically. The 6-hour reaction (early phase) was produced by very small doses of tuberculin, and, if sufficient amounts of protein were used, the early inflammation continued to increase *without a break* to give the usually observed manifestations (later phase). (That the early phase can be produced with less tuberculin than will elicit the later phase must be borne in mind when testing various tuberculins for qualitative activity.) The external characteristics of the early and later reactions are the same. All adults tested showed these early reactions, but in experiments with Dr. Elinor F. Downs newborn babies and about half of the young children tested failed to respond. By reading tuberculin tests early the percentage of recorded reactors would be greater, but the test would be no more specific since it was given by young children with active tuberculosis, some with primary calcified tuberculosis, and some with no chest lesions as shown by x-ray.

A SINGLE CULTURE MEDIUM FOR SELECTIVE ISOLATION OF PLASMA-COAGULATING STAPHYLOCOCCI AND FOR IMPROVED TESTING OF CHROMOGENESIS, PLASMA COAGULATION, MANNITOL FERMENTATION, AND THE STONE REACTION. George H. Chapman, Clinical Research Laboratory, New York.

The ingredients of the culture medium have a profound effect upon the cultural reactions of staphylococci. When conditions are most favorable for chromogenesis they are also, with minor adjustments, most favorable for coagulation of blood, mannitol fermentation, and the Stone reaction. The most satisfactory combination, in grams per liter, was mannitol, 10; agar, 15; gelatin, 30; tryptone, 10; anhydrous K_2HPO_4 , 5; lactose, 2; yeast extract, 2.5; and NaCl, 75. Incubation is for exactly 48 hours. Both *Staphylococcus albus* and *Staphylococcus aureus*, but no other bacteria, except "Streptobacillus," grow on the medium. Orange staphylococci rarely fail to clot blood and white colonies rarely clot blood,

whereas pale-colored colonies may or may not clot blood or ferment mannitol.

For testing for coagulase, emulsify a heavy loopful of growth in 0.2 ml of brain heart infusion and add 0.2 ml of satisfactory blood. Incubate up to 1 hour. For mannitol, put 1 drop of phenol red on a clump of colonies. For the Stone reaction, flood the plate with 5 ml of saturated ammonium sulfate and allow to stand 10 minutes.

PRODUCTION OF ANTIBIOTIC SUBSTANCES BY BASIDIOMYCETES. *W. J. Robbins, Annette Hervey, and Frederick Kavanauch*, Columbia University and New York Botanical Garden.

About 300 species in 42 genera of the Basidiomycetes were tested by the streak method against *Staphylococcus aureus* and *Escherichia coli*. Nearly two-thirds evidenced some activity against *S. aureus* and about one-third produced inhibitions of 10 mm or more. Forty-two of the more active Basidiomycetes were tested further by the disc method; 16 were moderately or strongly active against *S. aureus*. Twenty-five have been grown in liquid culture, and the antibacterial activity determined by the dilution method. Culture liquids active at dilutions of between 250 and 1,000 have been obtained from 6. The studies are being extended to other Basidiomycetes; efforts to concentrate and isolate the active materials, and tests of their effects on other organisms are underway. There is as yet no evidence that any of the substances are of therapeutic value.

ANTIBIOTICS FROM THE ASPERGILLI AND

PENICILLIA. *Charles Thom*, Port Jefferson, N. Y.

Inhibition of one organism by another in culture is a very old story to anyone who has been cultivating miscellaneous species together for the past forty years. Before the days of the pH apparatus we put litmus into the media, and saw how great a number of molds and bacteria would grow surrounded by broad zones of red free from other growths or partly so. Acid was thus our earliest recognized antibiotic though we did not use this word. Nearly all *Aspergilli* and *Penicillia* produce such acid reactions whenever specified sugars are used in the media. In a recent issue of *Science* (102, 627) the penicillins were shown to be a closely related series of organic acids having closely related clinical adaptability to control of specified bacterial diseases. There is a long list of organic acids—lactic, citric, gluconic, puberulic, penicillic, aspergillic, Kojic—and more. Each represents a form in which part of the substratum is broken down by one or many strains of fungi. Each of these is probably (some certainly are) metabolized in whole or in part by the fungus producing them. Perhaps some are discarded waste products. One may also safely say that all of them play some part in the defensive mechanism by which the organisms producing them survive in the intense struggle for existence which characterizes rotting masses in nature. The past four years have seen fabulous results in the study of penicillin. A start has been made with others. The possibilities open to further intensive study are not calculable.

VARIATION WITHIN STRAINS OF CLOVER NODULE BACTERIA IN THE SIZE OF NODULE PRODUCED AND IN THE "EFFECTIVITY" OF THE SYMBIOSIS

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Strains of *Rhizobium* may be distinguished in their behavior in symbiosis by their ability to infect a given host plant, as well as by the types and numbers of nodules produced and by the nodules' effectiveness in benefiting the host through nitrogen fixation. Allen and Baldwin (1931) have reviewed the changes which strains of *Rhizobium* have been observed to undergo, and the more important of these may be summarized. Nobbe and Hiltner (1893) and Frank (1899) claimed that growth of *Rhizobium* on gelatin media could induce ineffectivity in nitrogen fixation in the nodules or even complete loss of ability to infect the roots. Other workers have claimed that cultivation on nitrogen-rich media leads to a reduction in effectivity and vice versa, and Simon (1908), Snieszko (1929), and Hutchinson (1924) present evidence that culture in soil restores lost virulence and effectivity. Finally, Wünschik (1925) and Allen and Baldwin (1931) claimed that repeated plant passage led to changes in the effectivity of strains, although neither Stapp (1929) nor Virtanen (1945) was able to confirm Wünschik's results.

In all these cases it is not clear whether the observed changes were due to the selective action of the treatment on an already heterogeneous population or whether new variants had appeared during the experiments. The gradual changes often observed could thus be attributed either (1) to the selective increase of a small number of cells originally present in the culture, and bearing the newly observed character, (2) to the development of a few variants that subsequently increased differentially, or (3) to a general change occurring throughout the bacterial population. Further, the majority of tests were made as pot experiments, and under these conditions the possibility of contamination cannot be excluded.

The object of the present work was to study, with pure culture technique, the effects of plant passage and of various cultural environments in inducing variation in a population of *Rhizobium* known to be homogeneous at the commencement of the tests.

MEDIA AND METHODS

All stock cultures were maintained on a yeast-water agar medium and sub-cultured at about two-month intervals. The medium used had the following composition: mannitol, 1.00 per cent; K_2HPO_4 , 0.05 per cent; $MgSO_4$, 0.02 per cent; NaCl, 0.02 per cent; $CaCl_2$, 0.02 per cent; $FeCl_3$, 0.001 per cent; 10 per cent aq. yeast extract, 10 per cent; agar 1.5 per cent.

Throughout the experiments late-flowering Montgomeryshire red clover was used as the test plant. The seed was sterilized externally before planting by being washed for 3 minutes in 80 per cent alcohol, then for 3 minutes in 0.2 per cent mercuric chloride, followed by frequent washings with sterile water over a period of some hours. The sterilized seed was transferred with a wire loop to the surface of a test tube slope of "seedling agar" of the following composition: K_2HPO_4 , 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; $NaCl$, 0.01 per cent; $Ca_3(PO_4)_2$, 0.2 per cent; $FePO_4$, 0.1 per cent; $FeCl_3$, 0.001 per cent; agar, 1.2 per cent. The tubes containing the clover plants were kept in racks in a glasshouse and partially shaded on cloudless days. Development was best in conditions of good light but not under direct sunlight; the roots were shaded.

The plant cultures were allowed to grow under these conditions for 3 months or longer, depending on the time of year and the object of the experiment. At harvest, observations were made of the general appearance of each plant, whether large and dark green or small and pale yellow-green, and the length of all the nodules on the roots were determined; in some experiments dry weight and total nitrogen were also determined. Isolations of substrains from nodules were made from single colonies picked from platings of the contents of single nodules. Before plating, each nodule used for isolation was sterilized externally with alcohol and mercuric chloride as described for seed sterilization.

ORIGIN OF STRAINS A AND H.K.C.

Two original strains of *Rhizobium trifolii* were chosen for this study; an effective strain, strain A, obtained from Professor Bartel of Stockholm (referred to in some previous publications as strain "Bart. A"), and a local ineffective strain named H.K.C. Before the present investigation was commenced in 1939, the stock cultures of both strains and their early derivatives were under the observation of Dr. H. K. Chen. They were used frequently and for several years in studies in strain competition, effectivity, etc.; they were frequently plated, and during this time no changes in effectivity were observed.

In the course of the work rigid bacteriological control excluded contamination by other nodule bacteria, but at intervals the identity of each strain was checked serologically by Dr. A. Kleczkowski. A large number of substrains derived by plant passage from strain A were examined. These were named systematically on isolation, the name of each being derived from the parent strain by the addition of a digit. Thus substrains A151 and A152 were derived from substrain A15, which in turn was derived from substrain A1, which in turn was derived from strain A. It is realized that "substrain" may be regarded as too high ranking a term to distinguish reisolations which were in most cases indistinguishable, but it is retained for clarity.

The complete scheme of isolations made in the course of the first series of experiments on the stability of strain A is shown in figure 1, in which each circle represents a nodule produced by, and containing, the substrain numbered below it, and in which the diagrammatic section of a petri dish represents the selection of that substrain from a single colony. Thus the lowest circle represents a

nodule containing the original strain A, the contents of which were plated in December, 1938, yielding substrain A1. Six nodules containing this substrain were plated at different times, in different experiments, and from these six platings substrains A11 to A16 were derived. When a number of colonies were picked from the plating of the same nodule, such replicate isolations were all given the same substrain number.

THE STABILITY OF STRAIN A WITH RESPECT TO SIZE OF NODULES PRODUCED

The difference between effective and ineffective strains has been elucidated by Chen and Thornton (1940), who showed that, in the clover, pea, and soybean

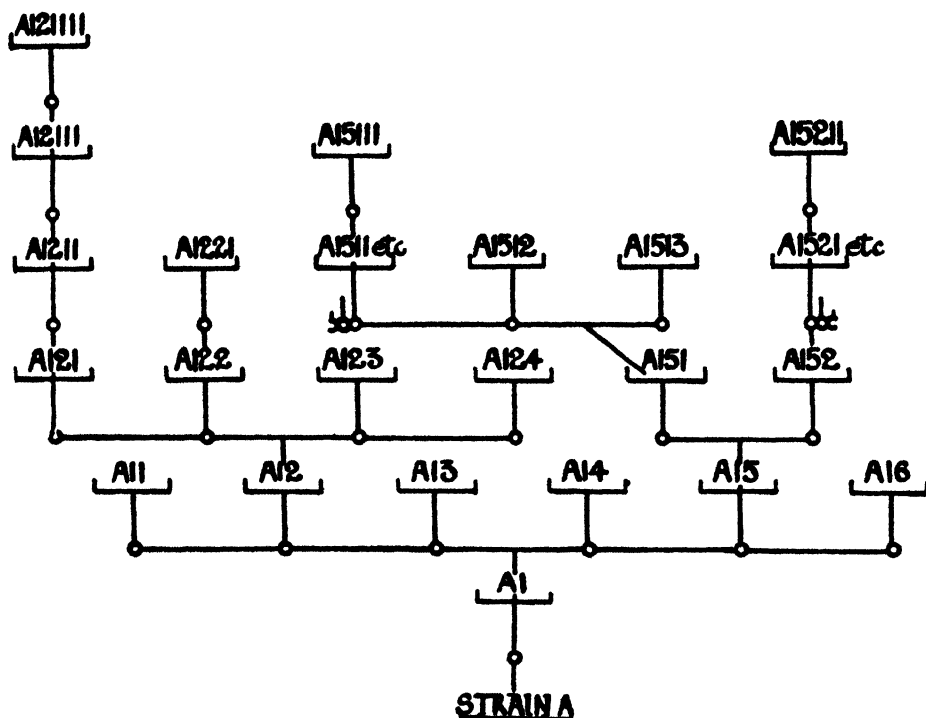


FIG. 1. RELATIONSHIP BETWEEN SUBSTRAINS DERIVED BY PLANT PASSAGE FROM STRAIN A

cross-inoculation groups, the effectivity of a strain does not depend on the efficiency of the nitrogen fixation process itself, but is a function of the mass and duration of the active bacterial tissue of the nodule. It was observed that clover inoculated with the effective strain A had relatively few nodules, ranging widely in size, whereas plants inoculated with the ineffective strain, Coryn, produced a large number of nodules all of which were small and which soon degenerated. Both strains fixed nitrogen at the same rate per unit mass of active bacterial tissue.

The great majority of clover strains conform in their nodulation broadly to the two types which were described by Chen and Thornton, and which are repre-

sented by the strains A and Coryn. Such a generalization does not hold for all strains under all conditions; not only do intermediate strains occur, but under some circumstances relatively large nodules are formed, which are nevertheless ineffective because the bacterial tissue of the nodule is very ephemeral. But any strain, whether or not it conforms to the A or Coryn type, has a characteristic distribution of nodule length when tested on a sufficient number of plants, and a departure from this would almost certainly be reflected in differences in effectivity. The distribution of nodule length was here used as a measure of change in effectivity, not only because it can be based on exact and well-replicated measurements, but also because this criterion has an intrinsic interest quite apart from its relation to effectivity.

Homogeneity tests of principal substrains in respect to nodule length. Studies in variation are apt to be invalidated by failure to ensure that the material under examination is homogeneous; in this instance, a bacteriologically pure line. The early plant passage substrains were therefore tested for their homogeneity in behavior in regard to size of nodules produced.

In the first two experiments the substrains A13 and A15, also A122 and A124, were tested and compared. The population of each was sampled by the plating and picking of about 30 random colonies, each colony being used to inoculate a pair of clover plants grown on an agar slope in a test tube. Each tube was planted with two clover seeds, though sometimes only one plant grew. At harvest a complete record of the nodule length distribution of each plant was made, from which was obtained the mean length of the nodules within each culture tube, i.e., produced from one picked colony. Similarly the average size of nodule produced by a substrain could be calculated as the mean length of all nodules produced by all the colonies picked from a plated nodule containing that substrain.

It was at once evident from an examination of the data of these experiments that the number of nodules and the distribution of nodule size varied greatly from plant to plant, and that the significance of small differences between substrains and between colonies was difficult to assess by simple comparison of mean values, so that recourse was had to statistical analysis. In each experiment independent comparisons were made between the mean nodule lengths in replicate tubes, each infected from a different colony, and between those of individual plants infected from the same colony. These comparisons are illustrated in figure 2. By this means the variance of nodule length within plants, i.e., between nodules on the same plant, was used to determine the significance of differences between duplicate plants in the same tubes; that between plants was utilized for testing differences between colonies, i.e., tubes; and the colony variance was used as an estimate for error in determining the significance of differences between substrains. The variances thus obtained in the second of these first two experiments and in some of the later experiments are given in table 1. In experiment 1, nodules containing substrains A13 and A15 were plated, and 24 random colonies of each substrain were tested on plants. These tests showed no significant difference in mean nodule lengths produced by the two substrains (8.58 ± 0.20 ,

8.27 ± 0.13 mm per 10, respectively), but complete analysis could not be made here as the individual plant results were not kept separate. In experiment 2, a similar comparison was made between substrains A122 and A124. Here, however, a complete analysis of variance was made, and it showed a significant difference only between duplicate plants in the same tube. The two substrains were similar, and each was homogeneous, no significant difference in behavior showing between isolations from the 69 colonies tested.

This and all later analyses emphasized that there was a significant difference between the nodule length distributions of individual plants in the same tube. This difference may have been due to factors inherent in the plant affecting response to homogeneous bacterial population, or to the development of bacterial

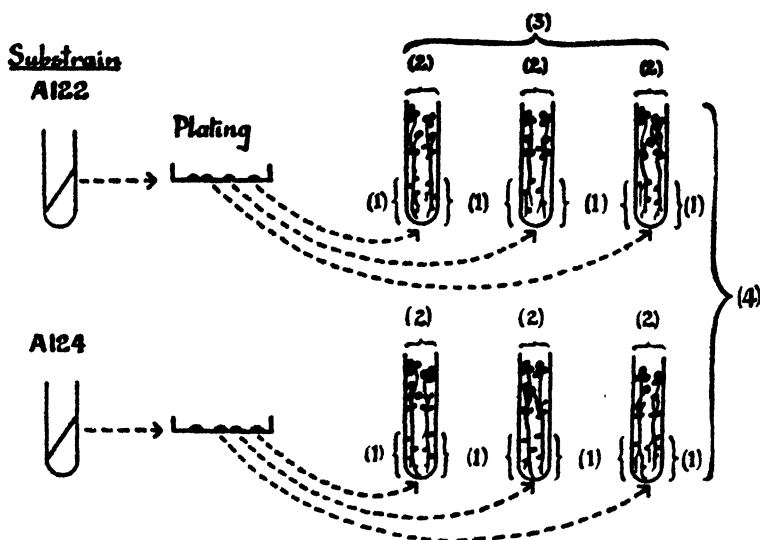


FIG. 2. COMPARISONS MADE IN THE ANALYSIS OF VARIANCE

- (1) Variance within plants.
- (2) Variance between plants in the same tube.
- (3) Variance between colonies (i.e., tubes).
- (4) Variance between substrains.

variants in or immediately around the individual plant, or to the selective infection of that plant by bacteria differing from the majority in the mean nodule length which they produce. On the last two views it should be possible to modify the mean nodule length by selection from large and small nodules, respectively, or from plants showing abnormal nodule length distributions. The effect of such selection was therefore tested.

Nodule selection experiments. Two plants infected with substrain A15 were chosen for the first test, experiment 3 (table 1). From the first plant having nodules of a mean length of 1.09 mm, a nodule 1.7 mm long was selected and plated, and from this plating 35 random colonies were picked (substrain A151), and each was separately tested on clover. The second plant bore nodules with

a mean length of 0.59 mm, from which a nodule of 0.5 mm was plated. Thirty-eight colonies were picked (substrain A152) and each was tested on clover. Analysis of variance showed no significant difference between the mean nodule lengths produced by the substrains isolated from the large and the small nodule, respectively. The comparison of replicate colonies showed that the bacterial contents of each nodule from which the substrains were isolated were also homogeneous (table 1, experiment 3).

In the second test (experiment 4), isolations were made from nodules on plants in experiment 3. Seven plants, each having few and large nodules produced by substrain A151, were selected, and from these 11 nodules ranging in length from 1.7 to 2.6 mm in length were plated. Thirty-one random colonies were picked from these plates and tested on clover (substrains A1511, A1512, etc). Two plants having many small nodules produced by substrain A152 were also selected, and from these plants 12 nodules 0.6 to 0.7 mm long were taken from the older roots, to avoid using young nodules that may later have grown larger. These nodules were plated and 27 colonies tested (substrains A1521, A1522, etc.). The mean nodule length produced by isolations from the large nodules was 0.845 mm; that produced by isolations from the small nodules was 0.802 mm. Analysis showed that these means were not significantly different, but the variance between isolates from different nodules on plants inoculated with the same substrain was less than that between replicate colony isolates from the same nodule. Thus, selection over two plant passages of large and small nodules borne on plants bearing, respectively, few large and many small nodules failed to alter the mean nodule length produced by the selected substrains.

However, the mean nodule lengths produced by replicate colonies from the same nodule in experiment 4 did show a variance significantly exceeding that between duplicate plants in the same tubes (table 1). This was attributable to two tubes in which both plants bore unusually large nodules. The colonies from which these tubes were inoculated came from different nodules inoculated with substrain A151, and the remaining colonies from the same two nodules produced nodules of normal length. Nevertheless, a large nodule from one of these tubes was plated, and 36 random colonies obtained from it were compared with 33 random colonies from a plating of substrain A1221, whose parent substrain, A122, had been shown in experiment 2 to produce a normal nodule length distribution. The isolates from this large nodule (substrain A15111) produced a mean nodule length of 1.079 ± 0.031 mm, which was not significantly different from that produced by substrain A1221, i.e., 1.107 ± 0.017 mm. There was thus no evidence that the abnormality in the two tubes of experiment 4 was due to any difference in the bacteria.

Similar negative results were also obtained in experiment 6, in which the contents of an abnormally large nodule containing substrain A1211 were plated and 35 colony isolates (A12111) compared in nodule formation with 35 from the ancestral substrain A1 (table 1).

The selection from large and small nodules, described above, took no account

TABLE 1
Analysis of variance of nodule length

EXPERIMENT	STRAINS	MEAN NODULE LENGTH (in 0.1 mm)	SOURCE OF VARIANCE	D.F.	SUMS OF SQUARES	MEAN SQUARE	F
2	A122	10.38	Between substrains	1	12.13	12.13	0.14
	A124	10.57	Between colonies	67	5,727.25	85.48	1.36
			Between plants	79	4,963.54	62.83	4.72 xxx
			Within plants	1,092	14,549.60	13.32	
	Total			1,239	25,252.52	20.38	
6	A1	8.37	Between substrains	1	22.91	22.91	0.50
	A12111	8.13	Between colonies	60	2,746.34	45.77	1.75 x
			Between plants	62	1,617.78	26.09	3.03 xxx
			Within plants	1,397	12,029.39	8.61	
	Total			1,520	16,416.42	10.80	
3	A151	8.06	Between substrains	1	16.76	16.76	0.59
	A152	7.89	Between colonies	71	2,021.58	28.47	0.99
			Between plants	100	2,879.99	28.80	3.59 xxx
			Within plants	1,961	15,731.57	8.02	
	Total			2,133	20,649.90	9.68	
4	A1511 etc	8.45	Between A151 and A152 lines	1	73.81	73.81	2.57
	A1521 etc	8.02	Between nodule isolates sister substrains	21	603.13	28.72	0.19
			Between colonies	35	5,344.87	152.42	5.81 xxx
			Between plants	58	1,520.78	26.22	3.42 xxx
			Within plants	1,445	11,089.73	7.67	
	Total			1,560	18,622.32	11.94	
10	A121111	10.50	Between substrains	1	253.60	253.60	0.81
	A121111W	9.58	Between colonies and between plants	28	8,738.73	312.09	20.25 xxx
			Within plants	1,193	18,378.83	15.41	
	Total			1,222	27,371.16	22.40	
11	A121111	10.67	Between substrains	1	1,878.20	1,878.20	343.36 xxx
	A121111W	7.43	Between colonies	16	87.51	5.47	0.04
			Between plants	6	898.38	149.73	55.66 xxx
			Within plants	371	998.09	2.69	
	Total			394	3,862.18	9.80	

of the degree of benefit shown by the plant; the plants from which the small nodules were selected did bear evidence of some nitrogen fixation. Occasionally, however, a plant was observed which showed the dwarf size, etiolation, red petioles, and the many small nodules characteristically produced by an ineffective bacterial strain. In one typical instance the mean nodule lengths of the aberrant and of the remaining normal plants in an experiment were found to be 0.533 ± 0.009 mm and 0.840 ± 0.010 mm, and in a second case 0.584 ± 0.010 mm and 1.124 ± 0.014 mm. In these and in other cases plants showing effective and ineffective responses appeared together in the same tube. From the first of these ineffective plants 34 colony isolations (substrain A16) were compared with the parent substrain A1 and with isolations from a large nodule (substrain A121111). The mean nodule lengths produced by each substrain were: A16, 1.045 ± 0.014 mm; A1, 1.025 ± 0.031 mm; and A121111, 1.092 ± 0.019 mm. They are not significantly different.

From the second ineffective plant infected with substrain A151, 20 colony isolations (substrain A1512) were compared with 20 from a large nodule on a normal plant (substrain A1513). The mean nodule lengths for A1512 were 1.108 ± 0.026 mm, and for A1513, 1.081 ± 0.026 . In both these experiments, therefore, the bacteria isolated from the plants that showed ineffective responses produced the mean nodule size and type of response typical of an effective bacterial substrain.

From these results it was concluded that the substrains derived from strain A since 1939 were very stable with respect to effectivity response and size of nodules. During the experiments outlined above 28 plant passage substrains were examined, involving the testing of about 600 colonies, the response of about 1,200 plants, and the measurement of more than 16,000 nodules. In this extensive series of tests no persistent change in the bacteria with respect to mean nodule length appeared, either during normal growth in the plant or as a result of deliberate selection.

Plant passage. The experiments summarized in figure 1 yielded some data on the absence of an influence of plant passage on effectivity, since most of these comparisons were made between substrains which had been passed through the plant for different numbers of times before testing. In these experiments the effects of 2, 4, 5 plant passages were tested, and in no case was any effect evident, a result contrary to the reports of Allen and Baldwin (1931), who described changes from effectivity to ineffectivity, and vice versa, on plant passage. It was thought that differences in experimentation might have been responsible for this disagreement, in that each plant passage culture was made in these experiments from a colony on a plating of the contents of a single nodule, so that an infrequent dissociative change would almost certainly have been overlooked. To test this possibility further experiments were undertaken using the stable effective substrain A121111 and the ineffective strain H.K.C., in an endeavor to follow Allen and Baldwin's technique and in addition to compare the effects of intervening plating and agar culture. The substrain A121111 was selected, not only because of its stability under laboratory conditions, but also because

there was no doubt of its bacteriological purity. The strain H.K.C. had not undergone so rigorous an examination as A121111 but had been under observation in the laboratory for a considerable time and was not known to have produced any forms differing in effectivity.

The transfers were conducted under sterile conditions using the agar tube technique of plant culture, and the isolates were tested in controlled test tube experiments. The series of plant passage transfers was begun in November, 1940, by inoculating seedling agar slopes, planted with red clover, with the two

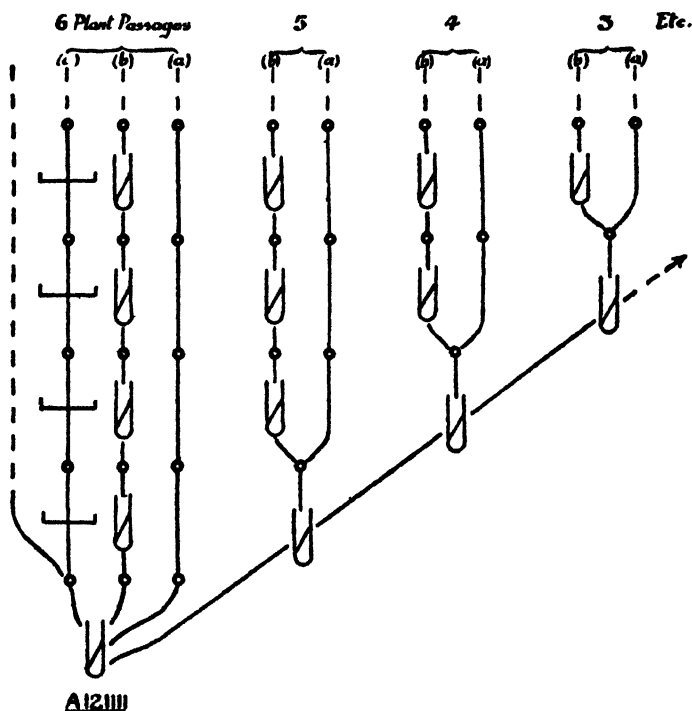


FIG. 3. SCHEME OF ISOLATION, PLATING, AND SUBCULTURE FOLLOWED IN PLANT PASSAGE EXPERIMENTS

strains. The scheme of isolations then followed will be made clear by reference to figure 3.

Three methods of plant passage were employed. (a) Six random nodules from the previous plant passage were crushed in saline, and the resulting suspension was used directly to infect the next plant passage. (b) A suspension from six crushed nodules was grown on an agar slope, incubated for 1 week, and then used as inoculum. (c) A similar nodule suspension was plated and a colony isolation used as inoculum. At each plant passage, fresh series using methods (a) and (b) were started from the stock cultures and continued to the end of the experiment. Thus, by September, 1941, substrains had been obtained that had been passed through the plant from 1 to 6 times by methods (a) and (b), including

one that had been passed 6 times with a plating between each passage. In addition, an isolation was made in September, 1941, from the original plant inoculated with the stock culture of A121111, which had been kept since November, 1940. This scheme was designed to test whether changes in the bacteria were produced by plant passage and whether these were lost during growth in agar or through the effects of plating.

The final isolates were each tested in 4 replicate agar tube cultures, each of which contained from 1 to 3 plants. After a period of 5 months of growth the nodules were measured as in previous experiments of this type, and, in addition, the dry weights of the individual plants and the total nitrogen content of half of each treatment (2 replicates) were determined. The results summarized in table 2 show no significant difference between the treatments. The control tubes remained without nodules.

In view of these negative results a further plant passage experiment was conducted; this was a continuation of the one described above in that the isolation of further plant passage inocula was made from the agar experiment just described. The number of plant passages was extended to eight, but final tests were only made on the fourth and eighth plant passage, and on the stock culture. The final effectivity test consisted of 10 replicate test tube cultures of seedling agar medium for each treatment, each tube planted singly with red clover. The seeds were sown in June and the experiment was concluded in October. The results are shown in table 2. Again no significant effects were produced by any of the methods of plant passage either as regards nodule length, plant dry weight, or nitrogen fixation. The culture obtained after 8 plant passages with intervening plating did not differ significantly in nodule length produced from the stock substrain A121111, which itself, after 5 previous similar passages, had been found not to differ significantly from its parent substrain A1. Thus 13 passages with intervening platings failed to alter the strain in respect to the character measured.

The negative results obtained by three different methods of plant passage make it difficult to explain the discrepancy between these results and those of Allen and Baldwin (1931) on grounds of technique. The difference may be in the greater homogeneity of the bacterial strains used in the present work. The evidence here presented shows that an originally homogeneous strain of *Rhizobium* does not undergo modification in its symbiotic behavior on repeated plant passage.

EFFECT OF STORING SUBSTRAIN A121111 IN SOIL

Appearance of an ineffective variant. The first part of this work provided no evidence that either plant passage or nodule selection could modify a genetically uniform strain, but it did provide, in the later plant passage derivatives of strain A, some material most thoroughly tested as to genetical homogeneity. It was decided to test the stability of this strain outside the plant. The first series of tests were made after storing substrain A121111 in Woburn soil.

Woburn sandy soil passed through a 0.5-mm sieve was distributed in 60-mg amounts in large (2-inch diameter) test tubes, which were plugged and sterilized

TABLE 2
Test of plant passage by three methods

METHOD OF PASSAGE	NUMBER OF PLANT PASSAGES	MEAN NODULE NUMBERS PER PLANT	MEAN NODULE LENGTHS PER PLANT	MEAN DRY WEIGHT PER PLANT	MG N PER PLANT
Test made Sept., 1941, to Feb., 1942. Substrain A121111					
			10/mm	mg	
(a) Direct plant pas- sage	(Stock) 0	15.3	12.2	30.3	1.26
	2	26.6 \pm 7.90	9.4 \pm 0.98	30.3	1.25
	3	20.8 \pm 4.30	10.1	30.0	0.93
	4	13.9	11.0	29.1	1.00
	5	13.6	11.3	26.4	0.85
	6	14.4	14.3 \pm 2.05	35.7 \pm 4.16	1.19
(b) Agar slope	2	21.0	11.4	33.6	1.05
	4	13.2 \pm 2.74	11.0	25.6 \pm 4.28	0.80
	6	17.6	9.5	27.6	0.86
(c) Plating	6	13.2	13.1	29.9	0.92
Test made June to October, 1942. Substrain A121111					
(a) Direct plant pas- sage	(Stock) 0	49.2 \pm 6.3		85.76 \pm 11.6	2.23
	4	60.0		100.70	2.47
	8	50.8		105.96	2.66
(b) Agar slope	8	70.8 \pm 10.2		88.0	2.34
(c) Plating	8	56.7		110.84	2.86
Test made Sept., 1941, to February, 1942. Strain H.K.C.					
(a) Direct plant pas- sage	(Stock) 0	41.7	9.0	8.85	0.17
	2	38.8	8.7 \pm 0.54	9.21	0.16
	4	42.4	9.8	8.60 \pm 1.98	0.16
	6	43.6 \pm 6.1	8.7 \pm 1.68	9.48	0.18
(b) Agar slope	2	42.6	8.7 \pm 0.55	8.58	0.17
	6	40.6	9.4	8.88	0.16
(c) Plating	6	36.3 \pm 4.4	10.0 \pm 0.50	9.75 \pm 0.87	0.16
Test made June to October, 1942. Strain H.K.C.					
(a) Direct plant pas- sage	(Stock) 0	103.6		30.42 \pm 1.99	0.42
	4	failed			
	8	failed			
(b) Agar slope	8	142.5 \pm 14.5		42.30 \pm 3.12	0.50 \pm 0.04
(c) Plating	8	102.7 \pm 13.6		33.04 \pm 3.93	0.44

in an autoclave by heating to 15 pounds for 1 hour on 2 successive days. Three of the tubes were given equal quantities of a thick suspension in sterile water of strain A121111 taken from a yeast agar slope, and three similar tubes of sterilized soil were kept without inoculation. These remained sterile. The tubes with soil were stored at room temperature in the dark for 6 months, by which time the soil had become dry and crumbly. Platings of the three soil samples were then made on yeast-water agar. The colonies that developed had an appearance similar to those typical of substrain A121111, and all appeared alike.

TABLE 3

Effectivity response and average nodule length of plants inoculated with isolations from soil cultures of substrain A121111

Test made May to July, 1941

Colonies showing effective response are placed above the line in the table and those showing ineffective response below

REPLICATE COLONY DESIGNATION	SOIL CULTURE NO. 1	SOIL CULTURE NO. 2	SOIL CULTURE NO. 3
	Average nodule length per plant	Average nodule length per plant	Average nodule length per plant
a	9.99	15.56	14.50
b	11.73	14.96	10.06
c	11.26	13.26	12.80
d	14.43	17.20	16.47
e	9.70	6.27	13.23
f	13.19	5.83	10.39
g	13.29	6.35	14.44
h	10.00	5.34	11.14
i	14.92	6.73	8.13
j	8.69	6.71	11.46
k	15.15	6.03	11.13
l	13.20	6.11	13.48
m	7.66	6.64	15.90
n	6.29	6.34	14.68
o	6.26	5.63	16.86
p			11.93
q			5.55

The average number of nodules on plants showing an effective response was 11.98.

The average number of nodules on plants showing an ineffective response was 55.48.

About 15 colonies derived from each soil sample were selected at random from each plate, and from each colony a loopful of bacterial growth was transferred to a tube containing red clover seedlings grown aseptically on the usual nitrogen-deficient agar medium. These tests on clover were kept from May to July, by which time the plants could be classified readily by eye as showing either an effective or an ineffective response. The plants fell into two clearly separate groups. Measurements of the nodules were made and these confirmed the clear-cut separation. Table 3 gives the mean nodule length found on plants

inoculated from every tested colony, the values below the horizontal line in each column referring to tubes showing ineffective responses. It is evident that clear-cut dissociation had taken place in the substrain A121111 during soil storage, giving rise to markedly ineffective variants. These appeared in all three tubes, though different proportions of variant to parent were found in each. Tests were then made of the contents of nodules on these plants. Subsequent investigation was most thorough in the case of soil culture no. 2, and description, illustrated by figure 4, will be confined to substrains from this soil, although data from soil cultures no. 1 and no. 3, as far as they went, confirmed the general conclusions.

Figure 4 shows, diagrammatically, the plant passage tests made with colony isolates from soil culture no. 2. A circle again represents the selection of a

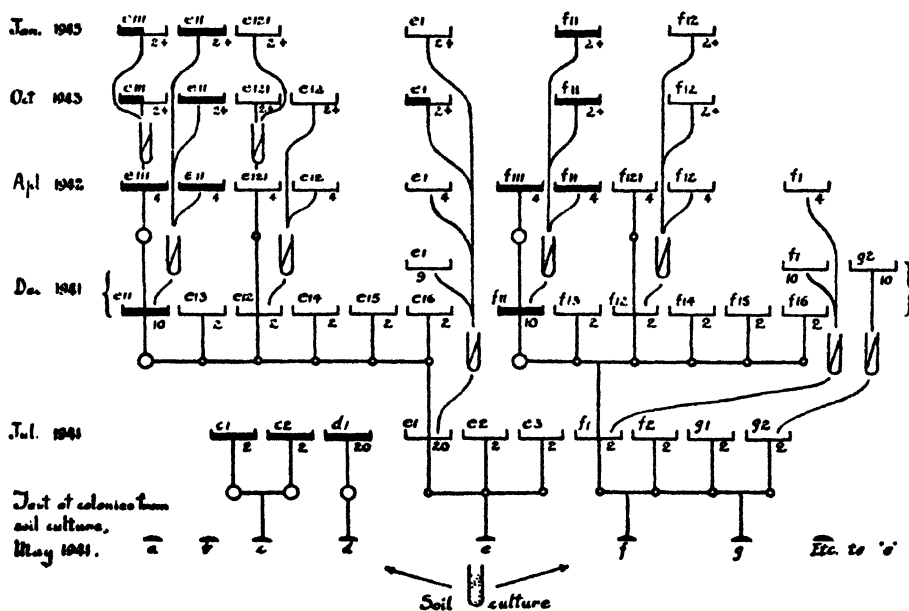


FIG. 4. THE DERIVATION OF SUBSTRAINS FROM A121111 AFTER STORAGE IN SOIL

nodule for plating, and the diagrammatic section of a petri dish indicates a test of replicate random colonies from a plating, the number of colonies tested being shown below. A thick base to the petri dish indicates that all the colonies tested gave effective responses, and a half-thick base, that the colonies differed in this respect. The designations of substrains bear the letter showing the soil culture colony from which they came, the numerals showing by the number of integers the number of subsequent plant passages. A diagrammatic agar slope indicates a stock culture.

Of the 15 colonies from soil no. 2 that were tested, 4, labeled *a* to *d*, produced effective nodules. Two nodules derived from colony *c* were plated, and 2 colonies from each nodule plating were tested, each on duplicate tubes of clover (table 4). One nodule from the colony *d* was also plated, and 20 colonies from this plating

were separately tested on clover; all these 24 colonies gave effective responses. The remaining 11 colonies from soil produced ineffective responses on the plants tested with them and also gave nodule length distributions that were typical of ineffective strains. Two nodules from plants inoculated from each colony from *e* to *m* were plated, and from each plating two colonies were picked and tested on clover (figure 4). An additional nodule from the tube inoculated with colony *e* was plated, and 20 colonies from this plating were separately tested on clover. Every one of these 54 colonies derived from 18 ineffective nodules gave a typical ineffective response when tested on the plant. These rather

TABLE 4

Tests of colony isolates from nodules of the first plant passage from soil culture no. 2 of substrain A121111

Test made July to November, 1941

SUBSTRAIN TESTED	NUMBER OF COLONIES PICKED AND TESTED	EFFECTIVITY	MEAN NODULE LENGTH PER PLANT	±
c1	2	E	10.97	0.90
c2	2	E	11.08	0.67
d1	20	E	11.43	0.58
e2	2	I	6.23	0.37
e3	2	I	5.52	0.02
e1	20	I	6.04	0.32
h1	2	I	5.71	0.16
i1	2	I	5.62	0.07
i2	2	I	5.57	0.24
j1	2	I	6.36	0.06
j2	2	I	5.85	0.15
k1	2	I	5.65	0.11
f2	2	I	5.79	0.54
f1	2	I	5.83	0.07
g2	2	I	6.14	0.46
g1	2	I	5.89	0.19
k2	2	I	5.90	0.08
l1	2	I	5.69	0.25
l2	2	I	6.93	0.51
m1	2	I	5.69	0.11
m2	2	I	5.56	0.21

extensive tests thus gave no indication of any mixture of effective parent and ineffective variant, either in the original colonies from soil or in the nodules produced when these original colonies were tested.

Serological identity of parent and ineffective variant. Subcultures made from the 54 colonies mentioned above, as well as from 16 colonies isolated from effective and ineffective plants inoculated from soils no. 1 and no. 3, were all tested serologically by Dr. A. Kleczkowski, who used an antiserum obtained against the original strain A. Equivalent agglutination occurred to a titer of 1:1,600 with all colonies, and with the strains A and A121111 used as controls. No agglutination occurred in any saline control. Cross-adsorption tests were also made

with the original effective strain and the ineffective dissociant, and no detectable differences were found. Full details of these experiments have been published (Kleczkowski and Thornton, 1944). These tests, together with the sterility of the original control soil cultures, completely excluded the possibility that the ineffective variant was a contaminant.

TABLE 5

Test of replated substrains from the first plant passage and of nodule isolates from the second plant passage after storing substrain A121111 in soil

Tests made December, 1941, to March 1942

SUBSTRAIN	NUM- BER OF COLO- NIES TESTED	MEAN NODULE LENGTH PER PLANT	±	OBSERVED EFFECTIVITY
Tested Dec., 1941				
First passage Substrain (stock)				
e1	9	6.21	0.186	All ineffective
f1	10	6.79	0.158	All ineffective
g1	10	6.26	0.115	All ineffective
Second passage Nodule isolate				
e11	10	8.94	0.381	All effective
e12	2	5.85	0.070	All ineffective
e13	2	6.32	0.089	All ineffective
e14	2	6.79	0.708	All ineffective
e15	2	5.92	0.240	All ineffective
e16	2	5.97	0.290	All ineffective
f11	10	8.38	0.985	All effective
f12	2	6.57	0.195	All ineffective
f13	2	6.07	0.195	All ineffective
f14	2	6.36	0.220	All ineffective
f15	2	6.31	0.046	All ineffective
f16	2	5.84	0.261	All ineffective
Tests made April, 1942, to July, 1942				
First and second passage Substrains (stock)				
e1	4	6.57	0.29	Ineffective
e11	4	11.70	1.36	Effective
e12	4	6.49	0.19	Ineffective
Third passage Nodule isolates				
e111	4	11.53	0.98	Effective
e121	4	6.97	0.99	Ineffective

Instability of the ineffective variant. In the two tests of the soil derivatives so far described the division into effective and ineffective types also showed as a clear distinction in the average nodule length per plant, but one plant infected with substrain e1 and one infected with f1 each bore one large nodule (2.9 mm and 2.4 mm in length) as well as numerous small nodules, with none of inter-

mediate size (figure 5). From both of these plants, platings were made from the large nodule and from five of the small nodules (figure 4). Twenty random colonies from each large nodule and two colonies from each small nodule were tested (substrains *e*11 to *e*16, and *f*11 to *f*16). Colonies from plated stock cultures of the parent substrains *e*1 and *f*1 were tested at the same time. The results are summarized in table 5. The parent substrains and all those derived from the small nodules (*e*12 to *e*16 and *f*12 to *f*16) produced ineffective responses. All the 20 colonies derived from the two large nodules produced effective responses (*e*11 and *f*11). A further test was then made by plating a large nodule containing substrain *e*11 and a small nodule containing substrain *e*12. Four colonies were tested from each plate (*e*111 and *e*121) as well as from stock cultures of the parental substrains. This test (table 5) confirmed the purity of both the large and the small nodule derivatives, both in regard to effectiveness and size of nodules produced. Nevertheless, when stock cultures of these five *e* substrains were retested in October, 1943, after a year's cultivation *in vitro*, substrain *e*111, originally effective, and *e*1, originally ineffective, then produced, on plating, mixtures of effective and ineffective colonies (figure 4). After a further long interval a test begun in January, 1945, using stock culture substrains of *e*1, *e*11, *e*111, and *e*121, as well as *f*11 and *f*12, confirmed these results by showing the liability of the variant substrains to revert in agar culture (figure 4).

INSTABILITY OF THE SUBSTRAIN A121111 STOCK CULTURE ON AGAR

The stock culture of A121111 was maintained on yeast-water agar and subcultured at intervals of 2 to 3 months. Throughout 1941-42 it was frequently used in experiments of all kinds, and it maintained its effectivity.

During this period, in April, 1942, a white colony dissociant appeared, the surface colonies of which differed from the normal type in presenting a very convex and often umbilicate or lobed surface, and instead of having a more or less mucilaginous texture, they were butyrous and could readily be lifted entire from the surface of the agar with an inoculating needle. The surface of the colony was smooth, but not so smooth as the normal type. The dissociant type was morphologically indistinguishable from the normal type. Israily and Sarygin (1930) have described what are probably similar rough variants, and Almon and Baldwin (1933) have also described variants in pigmentation and gumminess, but no studies of the effectivity of these variants were made.

The two types described above remained distinct when subcultured and later purified by plating twice. The new substrain was designated A121111W. It was found to be serologically identical with the parent substrain A121111.

Both strains were then tested on red clover in the usual way. A comparison of the mean nodule size produced by this variant and by its parent strain is given in table 1 (experiment 10). Analysis of the variance showed no significant difference between the behavior of the two strains. The mean dry weight of the plants inoculated with A121111 was 49.11 ± 2.86 mg, and that of the plants inoculated with A121111W was 44.00 ± 2.90 mg, a difference which again was

not significant. Thus in terms of dry weight and nodule size the effectivity of the dissociant was the same as that of the parent culture. The replated parent, A121111, was returned to stock, but again in November, 1943, it was observed on plating to consist of two colony types; the normal type and a dissociant type similar in all respects to A121111W. This was designated A121111W¹ and on serological examination was found to be indistinguishable from A121111, but on testing on clover was found to be ineffective (table 1, experiment 11).

A further ineffective variant appeared in the stock culture of A121111 early in 1944, but the behavior of this variant has not been studied in much detail. The relative numbers of effective and ineffective bacteria in the stock cultures of substrain A121111 were determined at intervals by testing random colonies. The proportion of the latter rose to about half by November 5, 1943, increasing during November and December and then disappearing. Simultaneous tests on plant passage isolates derived from substrain A121111 did not reveal any ineffective variants. Subsequent tests of the stock culture made at frequent intervals during 1945 have revealed no further ineffective variants.

DISCUSSION

The changes in character occurring in bacteria fall into two main classes. First there are those that are cyclical, as changes in cell form and structure appearing in such an order as to constitute a life cycle. These have been described in *Rhizobium* (Bewley and Hutchinson, 1920; Thornton and Gangulee, 1926) and can in fact be seen in the pleomorphism of all cultures of strain A and its derivatives. They are clearly distinguishable from the kind of changes here considered, which are of the type that persist for an appreciable time after the variant has been isolated and after it has been grown under the same conditions as its parent form.

Persistent changes again appear to fall into two groups. There are those in which a strain shows a progressive change, as when pathogenic virulence increases on host passage or is reduced by culture on laboratory media; and there are those sudden, and more or less permanent, changes in character which may appear either following a stimulus or without evident cause. It is to these sudden changes that the term "dissociation" is usually applied.

The apparent distinction between these last two types of variation is reminiscent of that formerly drawn between continuous variation and discontinuous variation or mutation in higher organisms, and it seems likely that an appearance of a gradual change in a bacterial population may be due to the selective increase of mutant forms in that population. Indeed, the work of Lincoln (1940), Gowen (1941), and Gowen and Lincoln (1942) on the rate of spontaneous and X-ray-induced dissociation in *Phytomonas stewartii* strongly suggests that bacterial dissociation in general is strictly analogous to mutation in higher organisms.

The genetical study of either progressive or dissociative variation is greatly complicated by the fact that the variant is usually observed not when it first occurs but only after it has grown for many cell generations in competition with

the parent form. The effects of plant passage upon a strain of *Rhizobium* recorded by Allen and Baldwin (1931) had the appearance of slow adaptive change, and because of the localized method of infection seemed to offer a chance of analyzing this type of variation.

In the present work, in which a bacterial strain of carefully tested phenotypic purity was used, neither plant passage nor selection was able to modify either the effectivity of the strain or the related character of mean nodule length. This would seem to exclude, at least in regard to the strains used, the occurrence of a slow progressive change in the bacterial population during passage through the host plant. But it does not necessarily exclude the occurrence of gene mutation in the bacteria giving rise to variants differing from the parent form in the size and effectivity of the nodule produced; and a closer examination of this possibility may be made on the basis of the results presented above.

It is evident that the readiness with which mutants which affect nodule size or effectivity can be detected differs greatly according to whether the mutation is in the direction of greater size and effectivity, or in the direction of smaller size and of ineffectivity. In the latter case, a parent strain normally producing effective nodules of large adult size will also produce nodules which are small because still young, so that an individual small nodule produced by a mutant can neither be distinguished at sight from a small young nodule produced by the parent strain, nor will its ineffectivity perceptibly influence the growth of the host plant. Such a mutation occurring in a nodule will thus be found only if the nodule in which it took place is selected for plating, and then only if the mutant form is selected in one or more of the resulting colonies picked and tested on the plant, upon which it will produce a smaller mean nodule size and give an ineffective response. Hence the chance of finding a mutation producing smaller nodules is limited by the number of colonies separately tested on the plant. During the experiments on plant passage and substrain selection, commencing with the effective strain A, more than 600 colonies were tested in this way. With any reasonable mutation rate, it is unlikely that gene mutations producing smaller nodules would have been detected, unless the mutant type increased considerably in relation to the parent type either in the colony or in the nodule. There was no evidence of such selective growth.

The likelihood of finding a variant in the direction of larger nodule size and effectiveness is, on the contrary, very much greater because it has a probability of being detected if it occurs either in the colony picked and tested, or in the nodule, since the presence of even a single nodule of abnormally large size will be readily seen among the small nodules produced by the parent strain, and a single effective nodule may perceptibly improve the growth of the plant (figure 5). Hence the chance of detecting an effective variant does not depend upon the number of colonies tested, but on the number of nodules produced and examined.

In the plant passage experiment using the ineffective strain H.K.C. a total of 2,896 nodules were examined. This number may well have been insufficient to afford a chance upon an effective variant, even supposing that a mutant change in a

single factor would render effective the strain H.K.C'. When, however, the ineffective variants produced by storing substrain A121111 in soil were passed through the plant, some 13,400 resulting nodules were examined, and among these two large nodules which were shown to contain effective organisms were observed. With a reasonable mutation rate, such a result would not be unexpected, and it may be legitimate to assume that here a reversion occurred in a single heritable factor in the bacteria.

The plant passage experiments as a whole are thus consistent with the view that bacterial mutants affecting nodule size and effectiveness occur during plant

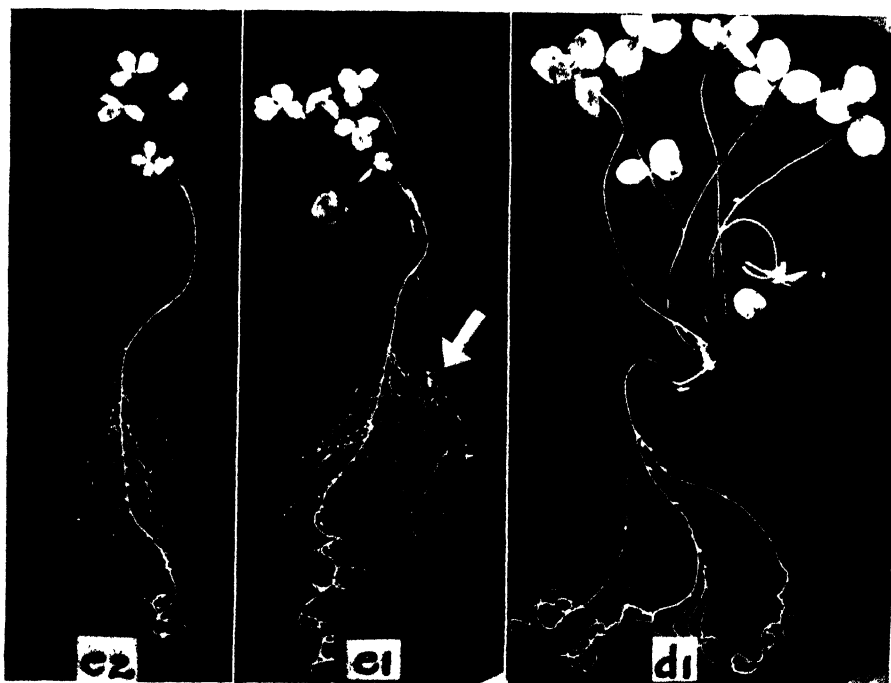


FIG. 5. CLOVER PLANTS INOCULATED WITH THE EFFECTIVE SUBSTRAIN *d1* AND THE INEFFECTIVE VARIANTS *c1* AND *c2*

The single large nodule produced by substrain *c1*, from which the effective reversion form was recovered, is indicated with an arrow.

passage, though there is no evidence that the plant environment selects bacterial variants in either direction. Plant passage is not a means of altering the efficiency of the bacteria. An absence of selection among the bacteria during plant passage may be related to their separation into microscopic colonies within the individual cells of the nodule, combined with the absence of suitable conditions for bacterial growth on the agar in which the plants were grown.

On the other hand, the high percentage of ineffective variant types producing small nodules, which were found after storing the effective substrain A121111 in soil, can best be explained by supposing that in the soil environment a strong selection occurred in favor of the ineffective variant.

It will be recalled that the ineffective form appeared in all three soil cultures, and from this it might be deduced that the conditions in the soil caused the dissociation. This is not necessarily the case. Each tube of soil was inoculated from the same mass inoculum from a slope culture of A121111, and it is probable that these enormous populations already contained numbers of mutant forms, some of which may have been relatively at an advantage in the new environment.

The storage in soil resulted in desiccation, which, like other unfavorable conditions, is a recognized "incitant" to dissociation insofar as it permits the survival of minority sections of the population. On yeast-water agar, even moderate desiccation leads to the death of bacteria. Their persistence in desiccated soil is probably due to the protective action of the soil colloids (Heller, 1941).

Changes in effectivity also occurred sporadically in culture on agar and possibly in the medium in the neighborhood of the plant root and in the nodule itself, but the similarity of the results of the tests made in 1943 and 1945 of stock cultures of *e1*, etc., show that the factors influencing the multiplication of the variant do not necessarily cause it to supplant completely the parent forms, or even to become dominant. Information is needed on the nature of the equilibrium existing in such heterogeneous populations, as well as on the particular soil conditions apt to encourage the emergence of ineffective variants, and on the relative stability in soil of different strains of *Rhizobium*.

These results have an important bearing on the practice of legume seed inoculation in emphasizing the need for frequent tests of the effectivity of cultures issued for this purpose in order to guard against the development of undesirable variants.

It will also be recalled that statistical analysis of the data revealed great variation in the response of individual clover plants to the same strain of bacteria, in some cases to an extent involving complete ineffectivity in the response of individual plants with the normally effective strain A. This plant variability is being investigated on genetical lines.

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The author wishes to record his indebtedness to Dr. H. G. Thornton, F.R.S., for much helpful advice and valuable criticism during the course of the experimental part of the work, and in the presentation of the results. Thanks are also due to Dr. H. K. Chen for the isolation of the initial plant passage substrains, and to Dr. A. Kleczkowski for kindly undertaking the serological examination of the mutant substrains.

SUMMARY

The paper describes experiments on the influence of passage through the host legume and of storage in soil and on agar medium upon the symbiotic behavior of two strains of *Rhizobium*. The results refer to mean length and "effectivity" of nodules on red clover grown under bacteriologically controlled conditions.

The original culture of the effective strain A was found to be uniform in the

behavior of isolations from replicate colonies, although considerable variation was found between the responses of individual plants infected from the same colony.

Neither plant passage with or without intervening plating or short-time culture on agar nor selection from large or small nodules had any effect on the mean size or effectivity of the nodules produced by strain A.

Plant passage similarly failed to modify the behavior of the ineffective strain H.K.C.

On the other hand, after the storage of strain A in sterilized Woburn sandy soil ineffective variants were found to constitute a considerable proportion of the bacterial population. These variants resembled the parent type in cultural and serological characters.

After the passage of these ineffective variants through the plant, two reversions to the effective parent type were found among the 13,400 nodules examined. These remained effective on further plant passage.

Stock cultures on agar slopes, both of the effective parent type and of the ineffective variant, showed an occasional tendency to produce new variants in effectivity. Variants in type of growth on agar also appeared under these conditions.

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AN ADDITIONAL SHIGELLA PARADYSENTERIAE SEROTYPE¹

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In recent years new *Shigella* strains have been reported by several investigators. Boyd (1940) described a number of *Shigella paradyserteriae* serotypes from India, many of which were found to have wide distribution. Working with microorganisms isolated in India and the Middle East, Sachs (1943) found *Shigella* cultures belonging to the group which do not attack mannitol. The occurrence in India of non-mannitol-fermenting microorganisms which differed antigenically from both *Shigella ambigua* and *Shigella dysenteriae* was mentioned also by Boyd. Two such strains of *Shigella* were studied in North Africa (vicinity of Casablanca and Bizerte) by Christensen and Gowen (1944). Type A and type B of Christensen and Gowen were found to be identical with two non-mannitol-fermenting *Shigella* strains isolated by the writer near Oran, Algeria, in September, 1943, and identical with *Shigella* sp., Sachs Q771 and Q1167, respectively. MacLennan (1944) and others also studied members of this group in the Mediterranean area. *Shigella* strains which were similar to those described by Sachs were reported from the United States by Gober *et al.* (1944).

In the course of our work in the Mediterranean area during the past 2 years a number of microorganisms were encountered which differed serologically from known *Shigella* strains. The purpose of this communication is to present the results of an investigation of one of these—a *Shigella paradyserteriae* by virtue of its biochemical reactions but serologically distinct from recorded types. In the following pages this serotype will be referred to as *Shigella paradyserteriae*, Lavington I.

SOURCES OF CULTURES

Since the history of the isolation of this microorganism is somewhat involved, it is presented in detail so that possible confusion in the literature may be avoided.

Apparently the Lavington I serotype first was isolated in Casablanca during May and June, 1943, by Major A. H. Stock of the 2nd Medical Laboratory (Stock, 1945). The microorganism occurred in epidemic proportion in a United States Army installation and was isolated from the feces in 214 cases of bacillary dysentery. Although the cultures presented biochemical characteristics of *Shigella paradyserteriae*, they did not agglutinate in any of the available antisera. It was concluded by Ernst and Stock (1943) that the microorganism was probably an additional serotype. In January, 1945, Stock submitted his type T culture to this laboratory for comparison with our unclassified cultures. A small quan-

¹This investigation was made in the Bacteriology Section of the 15th Medical General Laboratory, United States Army, located at Naples, Italy.

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TABLE 1
Sources of cultures

CULTURE NUMBER	SOURCE	LOCALITY	ISOLATED BY OR RECEIVED FROM
2-2-4 (Type T)*.....	Patient, American	Casablanca	Stock, 2nd Med. Lab.
Lavington I†.....	Patient, American	England	Francis, Emergency Vaccine Lab.
Lavington I*†.....	Patient, American	England	Heller, 1st Med. Gen. Lab.
6900.....	Patient, American	France	Francis
8944*.....	Patient, American	France	Heller
8945.....	Patient, American	France	Heller
2-3-417.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-476*.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-723.....	Patient, American	S. Italy	262nd Station Hosp.
2-3-724.....	Patient, American	S. Italy	262nd Station Hosp.
2-3-931*.....	Patient, American	S. Italy	262nd Station Hosp.
2-3-1008.....	Patient, American	S. Italy	262nd Station Hosp.
2-3-1009.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-1028.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-1029.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-1124.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-3-1126†.....	Patient, Italian	S. Italy	15th Med. Gen. Lab.
2-4-318.....	Patient, American	S. Italy	15th Med. Gen. Lab.
2-4-605.....	Patient, American	N. Italy	2nd Med. Lab.
2-4-607.....	Patient, American	N. Italy	2nd Med. Lab.
2-4-608.....	Patient, American	N. Italy	2nd Med. Lab.
2-4-613.....	Patient, American	N. Italy	2nd Med. Lab.
2-4-930†.....	Carrier, Italian	S. Italy	15th Med. Gen. Lab.
2-5-461.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-5-978.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-5-1037.....	Patient, American	N. Italy	2nd Med. Lab.
2-5-1224.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-6-1415.....	Patient, American	?	U. S. Hospital Ship
2-6-1433.....	Patient, American	S. Italy	300th General Hosp.
2-7-643.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-7-1501.....	Food handler, German	S. Italy	15th Med. Gen. Lab.
2-7-1714.....	Patient, American	S. Italy	15th Med. Gen. Lab.
2-7-1727.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.
2-8-209.....	Food handler, Italian	S. Italy	15th Med. Gen. Lab.

* Cultures were used for antiserum preparation.

† Both Lavington I cultures originated in Dr. Joan Taylor's laboratory.

‡ Two cultures were isolated from the same individual.

tity of antiserum made with the type T culture in 1943 was also supplied. Stock's type T is 2-2-4 in our series.

During early March, 1945, an antiserum (labeled Lavington I) prepared with a new *Shigella paradysenteriae* type was received from Lieutenant-Colonel A. E. Francis, R.A.M.C., of the Emergency Vaccine Laboratories, East Everleigh, England. Francis received cultures of the new type from Doctor Joan Taylor, of Oxford, who isolated it in England (20 cases) and from Captain George Heller, of the 1st Medical General Laboratory, who recovered a number of cultures from cases in France. Francis (1945) found that Taylor's Lavington I and Heller's type culture (6900) were identical, as shown by reciprocal adsorption tests.

The Lavington I and strain 6900 cultures were received from Francis during May, 1945, as were cultures 8944 and 8945 from Heller. Cultures 2-4-605, 607, 608, 613, and 2-5-1037 were isolated from cases in northern Italy in the vicinity of Florence and Modena by Stock and personnel of the Second Medical Laboratory during the latter part of 1944 and the spring of 1945. The remaining cultures were isolated by various United States Army hospital laboratory workers and sent to this laboratory for identification (7 cultures), or were originally isolated here (16 cultures). The sources of individual cultures are listed in table 1.

BIOCHEMICAL STUDIES

The cultures isolated here were recovered on SS agar (Difco), but Stock's were picked from desoxycholate citrate agar (D.C., Baltimore Biological Laboratory). The colony form on these media, on eosin methylene blue, and on plain agar plates conforms to the descriptions recorded for other dysentery bacilli. Variation of the S to R type was noted in most of the cultures, although no completely rough forms were seen. Small colony variants appeared in some cultures, but these were not studied in detail.

Carbohydrate media were prepared by adding Seitz-filtered solutions of the various test substances to an autoclaved basal medium made up of beef extract (0.3 per cent), peptone (1.0 per cent, bacto), sodium chloride (0.5 per cent), and Andrade's indicator. The final pH of this medium was about 7.2. Glucose, lactose, sucrose, and glycerol were each employed in 1.0 per cent concentration. The other carbohydrates were used in a concentration of 0.5 per cent. Simmon's citrate agar (Difco) was employed in tests for citrate utilization. The Voges-Proskauer and methyl red tests were done with 24- and 48-hour cultures, respectively, grown in MR-VP medium (Difco). A 40 to 50 per cent (weight per volume) potassium hydroxide solution containing 0.3 per cent creatine was employed in the tests for acetylmethylcarbinol production. Indole tests were made by two methods. The first was the Gnezda test in which filter paper strips were impregnated with a saturated solution of oxalic acid, dried, and suspended over 2.0 per cent peptone water. The alternate method for indole was the standard 1.0 per cent tryptone water culture tested after 24 hours' incubation with Kovac's reagent. Tests for the production of hydrogen sulfide were made with the aid of strips of filter paper soaked in a saturated solution of neutral

lead acetate, dried, and suspended over the 2.0 per cent peptone water cultures. Many *Shigella* strains produce blackening of such papers.

Motility tests were made with two media, the first of which is a modification of the Jordan *et al.* medium used by *Salmonella* investigators both to determine motility and to separate phase variants (Edwards and Bruner, 1942). The alternate motility medium was prepared by adding 0.25 per cent agar to 1.0 per cent tryptone water. Both motility media were inoculated with a loop at the top of the medium column.

Tests for urease production were made with a medium devised by Christensen (1944). Since the formula for this medium has not to our knowledge been published, it will be included here: distilled water 1 liter, glucose 1.0 g, peptone (Difco) 1.0 g, sodium chloride 5.0 g, monobasic potassium phosphate (KH_2PO_4) 2.0 g, phenol red 0.012 g, and agar 20 g. The pH is adjusted to 6.8 to 6.9, and the medium is sterilized in the autoclave. When the basal medium has cooled to 50 to 55 C, 80 ml of a Seitz-filtered 20 per cent urea solution are added, and the medium is tubed in approximately 3-ml quantities in Loeffler tubes and allowed to solidify so as to have a deep butt and a short slant. A heavy inoculum is made on the slant without stabbing. Although some *Aerobacter* cultures may yield a positive or doubtful reaction within 18 to 24 hours at 37 C with this medium, as compared to a delayed trace or entirely negative reaction with the highly buffered medium of Rustigian and Stuart (1941), no *Shigella* or *Salmonella* species thus far tested has given even a trace reaction after prolonged incubation. The Christensen medium is an exceptionally valuable one in routine work for the early recognition of *Proteus* and paracolon *Aerobacter* cultures. All cultures which yield an acid butt and an alkaline slant on Kligler's medium (modified with added sucrose), regardless of gas or blackening from hydrogen sulfide, are inoculated onto the urea medium. Cultures which are positive at a preliminary reading made after 3 to 4 hours' incubation at 37 C or which show trace or doubtful reactions following overnight incubation are eliminated from subsequent serological and biochemical tests. Used in this manner the urea medium has aided materially in the examination of thousands of feces specimens. For the differentiation of *Proteus* cultures, however, the medium of Rustigian and Stuart is essential.

Tests for the reduction of trimethylamine oxide were made by the method of Wood and Baird as outlined by Weil and Black (1944). The tests were performed with 24-hour cultures on two separate occasions with two lots of medium. Appropriate positive and negative controls were included on each occasion: *Shigella sonnei* and *Shigella dispar* cultures were positive, and microorganisms of the *S. paradysenteriae* Flexner group (I through VI) were negative, as were several cultures of *S. dysenteriae*.

All biochemical tests were incubated at 37 C with the exception of motility tests, which were incubated at about 28 C on top of the incubator (7 days). Negative carbohydrate media were held for periods varying between 28 and 38 days. Gelatin and citrate tubes were kept for at least 21 days, the gelatin being

tested for liquefaction at intervals of 4 or 5 days. The final reading of the urea medium was made after 7 days' incubation.

The biochemical and physiological reactions of the first 27 cultures listed in table 2 were studied in some detail. The remaining 7 cultures were tested with only those substrates customarily employed in the identification of dysentery bacilli.

All cultures were nonmotile, methyl-red-positive, and Voges-Proskauer-negative. Sodium citrate was not utilized, gelatin not liquefied, nor urea hydrolyzed by any of the cultures. Indole was produced by all cultures, and tests were positive by both methods. Three cultures (2-4-605, Lavington I, received from Francis, and 6900) produced a slight yellow-brown discoloration of the lead acetate papers after 48 hours' incubation; the other cultures were entirely negative.

Acid but not gas was produced within 24 hours from glucose, mannitol, arabinose, and xylose by all cultures. As indicated in table 2, maltose utilization was delayed. All cultures produced a small amount of acid in maltose broth on the first to the fourth day as evidenced by a pink coloration. Further incubation resulted in eventual strong acid production with a bright red medium in 3 to 35 days. Had a 21-day observation period been used, several of the cultures would have been recorded as negative or as doubtful. Sealing the maltose tubes with corks dipped in paraffin tended to hasten the reaction in most cases. Strong acid reactions were obtained after 2 to 12 days' incubation with 27 cultures so treated. There was one case in which acid was produced from maltose on the fifth day in both the sealed and unsealed tubes; also there was an instance in which a longer period was required in the sealed tube. The 27 cultures were retested in maltose broth, pH 7.4, with bromcresol purple as the indicator. The weak acid reactions were not noted with this indicator, but definite reactions were apparent after 3 to 12 days' incubation, the majority of cultures requiring 9 to 11 days.

Six cultures (table 2) fermented sorbitol within 24 hours, sixteen required 48 hours, and the remaining cultures required 7 to 8 days before strong acid reactions occurred. Weak reactions were noted in sorbitol broth in 1 to 5 days; these became strongly acid upon further incubation. Four cultures (table 2) that were negative by the usual method of testing produced acid reactions in sealed tubes. All cultures failed to ferment lactose, sucrose, salicin, dulcitol, rhamnose, and adonitol after prolonged incubation periods of 28 to 38 days at 37 C.

Acid without gas was produced within 24 hours from trehalose, galactose, and fructose by 27 cultures tested on these substrates. Likewise, acid reactions occurred in glycerol broth after 6 to 13 days. Slight acid production was noted in glycerol on the third to the sixth day of incubation. This same group of 27 cultures failed to ferment inositol and raffinose. Cellobiose was not utilized in 38 days by any of 25 cultures tested on it. The group of 27 cultures reduced trimethylamine oxide. Two cultures (m 280 and 8-770) of *S. paradyseutariae*, Boyd V (P.143), were tested at the same time as the group of 27 cultures of the

TABLE 2

Biochemical reactions of Shigella paradysenterias, Lavington I, cultures

CULTURE	GLUCOSE, MANNITOL, XYLOSE, ARABINOSE	LACTOSE, SUCROSE, SALICIN	MALTOSE	SORBITOL	RAMNOSE, ADONITOL, DULCITOL	INDOLE M.R.	CITRATE V.P.	MOTILITY GELATIN UREA
2-2-4 (Type T) ...	A	—	(A)	—	—	+	—	—
Lavington I.....	A	—	(A)	(A)	—	+	—	—
Lavington I.....	A	—	(A)	(A)	—	+	—	—
6900.....	A	—	(A)	(A)	—	+	—	—
8944	A	—	(A)	(A)	—	+	—	—
8945.....	A	—	(A)	(A)	—	+	—	—
2-3-417.....	A	—	(A)	(A)	—	+	—	—
2-3-476.....	A	—	(A)	(A)	—	+	—	—
2-3-723	A	—	(A)	(A)	—	+	—	—
2-3-724..	A	—	(A)	(A)	—	+	—	—
2-3-931	A	—	(A)	A	—	+	—	—
2-3-1008.....	A	—	(A)	(A)	—	+	—	—
2-3-1009.....	A	—	(A)	—	—	+	—	—
2-3-1028.....	A	—	(A)	—	—	+	—	—
2-3-1029	A	—	(A)	(A)	—	+	—	—
2-3-1124.....	A	—	(A)	A	—	+	—	—
2-3-1126.....	A	—	(A)	(A)	—	+	—	—
2-4-318.....	A	—	(A)	(A)	—	+	—	—
2-4-605.....	A	—	(A)	(A)	—	+	—	—
2-4-607.....	A	—	(A)	A	—	+	—	—
2-4-608.....	A	—	(A)	A	—	+	—	—
2-4-613.....	A	—	(A)	(A)	—	+	—	—
2-4-930.....	A	—	(A)	A	—	+	—	—
2-5-461	A	—	(A)	(A)	—	+	—	—
2-5-978.....	A	—	(A)	(A)	—	+	—	—
2-5-1037.....	A	—	(A)	(A)	—	+	—	—
2-5-1224.....	A	—	(A)	(A)	—	+	—	—
2-6-1415.....	A	—	(A)	(A)	—	+	—	—
2-6-1433.....	A	—	(A)	A	—	+	—	—
2-7-643.....	A	—	(A)	(A)	—	+	—	—
2-7-1501	A	—	(A)	(A)	—	+	—	—
2-7-1714.....	A	—	(A)	—	—	+	—	—
2-7-1727.....	A	—	(A)	(A)	—	+	—	—
2-8-209.....	A	—	(A)	(A)	—	+	—	—

() indicates delayed reactions, 48 hours or more.

Lavington I type. Except for the fact that neither culture fermented maltose (30 days) and one culture failed to produce acid from glycerol, the results were identical with those listed for the Lavington I type (table 2).

SEROLOGICAL STUDIES

Methods. In addition to those received from Francis and Stock, antisera were prepared with the Lavington I, 8944, type T (2-2-4), 2-3-476, and 2-3-931 cultures (table 1). Cultures selected for immunization were plated, and smooth colonies were picked to infusion agar slants in 16-by-160-mm culture tubes. After incubation for 20 to 24 hours the slants were tested for smoothness in 1:500 acriflavine, and in 1:10 Lavington I antiserum for agglutinability. The growth was removed with formalinized (0.3 per cent of 40 per cent formaldehyde) solution, 10 ml per slant. Vaccines so prepared were injected into rabbits at 3- to 4-day intervals in doses increasing from 0.5 ml to 5.0 ml. The first dose was administered subcutaneously and subsequent doses were given intravenously. The animals were test-bled a week following the last injection, and, if the titer was found to be satisfactory, the animals were bled from the femoral artery. Antisera were preserved with a mixture of equal parts of ethyl ether and phenol, 0.009 ml per 1.0 ml of serum.

Bacterial suspensions for agglutination were prepared in the manner employed by many investigators (e.g., Wheeler, 1944). Antisera were diluted serially, 1:20 to 1:5,120, in the usual manner, and all tests were read with the unaided eye following overnight incubation in a water bath at 50 to 52 C.

Antisera to be adsorbed were diluted 1:10 and added to an adsorptive dose calculated to be slightly in excess of that required. Adsorbing suspensions remained at 37 C for 20 to 22 hours; after this they were placed in the icebox for 24 hours. An adsorbing dose consisting of the growth from two 90-mm plates of infusion agar (20- to 22-hr cultures) effectively removed the homologous agglutinins from 1.0 ml of 1:10 antiserum. There were two exceptions to this, in which cases a larger adsorbing dose was necessary.

In the experiments designed to determine the serological relationship of the Lavington I cultures to known *Shigella* strains, preliminary tests were made employing only three dilutions of antiserum, 1:50, 1:100, and 1:200, and a control. If positive or doubtful results were obtained with any antiserum, that antiserum was diluted serially and the suspension retested.

Results. (1) It was noted that the Lavington I antiserum supplied by Francis as well as the type T antiserum from Stock agglutinated antigens 2-2-4 (Stock's type T), Lavington I, 8944, and our culture 2-3-931 to titer. It was then established that adsorption of the Lavington I antiserum or of type T antiserum by any one of the cultures removed all agglutinins for the homologous culture.

(2) Antisera were made with cultures isolated in Africa (2-2-4), England (Lavington I), France (8944), and Italy (2-3-931). These four sera and their homologous cultures were used in another group of adsorptive tests. Master dilutions were made with each of the four unadsorbed antisera, and the dilutions were tested with the homologous and three heterologous cultures. Each antiserum agglutinated the four suspensions in dilutions of 1:2,560 to 1:5,120.

Each of the four antisera was then adsorbed with four cultures, the homologous

and three heterologous. The 16 adsorbed sera thus obtained were then tested with the same four cultures. The results of these 64 tests were negative.

(3) An antiserum prepared with culture 2-3-476 (table 1) was employed in a third series of adsorptive tests to ascertain whether or not any differences could be noted between the various cultures isolated in Italy and elsewhere. Antiserum 2-3-476 agglutinated all cultures in a dilution of 1:5,120, and a few cultures exhibited weak reactions at 1:10,240. This antiserum was adsorbed with the first 24 cultures listed in table 1. Each of the 24 adsorbed sera thus obtained was tested with suspensions of the homologous (2-3-476) culture and the adsorbing culture. In each case the adsorbing culture removed all agglutinins from the antiserum.

(4) Studies designed to reveal the serological relation of the Lavington I cultures to other *Shigella* types were undertaken. Preliminary tests had indicated that the microorganisms bore little or no serological relationship to previously described types.

Formalinized suspensions were prepared in bulk with cultures 2-3-476, Lavington I, 2-2-4, and 8944. These suspensions were tested for agglutination in three dilutions (1:50, 1:100, 1:200, and control) with 41 unadsorbed *Shigella* antisera. Readings were made after overnight incubation in a water bath at 50 to 52 C.

No agglutination occurred with antisera of the *Shigella paradyserteriae*, Flexner group, types I through VI, in any dilution tested. Three Flexner II antisera were employed. Likewise, negative results were obtained in antisera prepared with members of the *S. paradyserteriae*, Boyd group: Boyd I (170), Boyd II (P.288), Boyd III (D.1), Boyd IV (P.274), Boyd V (P.143), and Boyd D.19 (Boyd VI). Two Boyd V antisera were employed. No agglutination occurred in an antiserum induced by culture 2-193 (*S. paradyserteriae* serotype with strong serological relation to members of the Boyd group, especially Boyd IV and V, and some Flexner group relationship—unpublished data; Francis, 1945; Wheeler, 1945). The *Shigella alkaescens* antiserum employed was one prepared with culture 1-2 which has antigens A, B, C, D (Stuart, 1944). No relationship to *S. alkaescens* was detected.

The four suspensions were also tested in antisera made with five microorganisms which have the biochemical reactions of members of the *S. paradyserteriae* group but which differ serologically from established types. The four Lavington I cultures were unagglutinated by these antisera in three dilution tests.

No significant relationship between the Lavington I and the lactose-positive *Shigella* cultures was detected. The four suspensions were tested with 1:50, 1:100, and 1:200 dilutions of three *Shigella sonnei* antisera (two S and one R) and with six *Shigella dispar* antisera. The latter included types I, II, III, and IV (Carpenter, 1943, 1944), a *Shigella madampensis* antiserum supplied by the Army Medical School, and an antiserum made with culture 10-888, isolated in Italy. Culture 10-888 bears some serological relationship to Carpenter's type II.

The 1:50 dilution of *S. madampensis* and one of the *S. sonnei* (S, 5-493) antisera were doubtful, but it was found that when these sera were diluted serially 1:20 to 1:1,280 a doubtful reaction occurred in the 1:20 but not in the higher dilutions.

Antisera prepared with *Shigella* sp., Sachs Q771, Q1167, Q1030, Q454, Q902, and J.P.6 were tested with suspensions of the four Lavington I cultures. A doubtful result was obtained in the 1:50 dilution of the Q771 antiserum but not in the two higher dilutions. This antiserum was diluted serially and the suspensions retested. The repeated tests were doubtful at 1:20 but were negative in all higher dilutions. The Lavington I cultures were negative in the other five *Shigella* sp., Sachs, antisera in all dilutions of the three-tube test. Antisera received from Francis against the two motile Sachs types (B.81 and B.105) also failed to agglutinate the Lavington I cultures.

Lastly, the Lavington I suspensions were tested with a *S. dysenteriae* antiserum furnished by the Army Medical School and with a *S. ambigua* serum. Agglutination was not apparent with these antisera in the dilutions employed.

(5) The next procedure investigated was the converse of the tests just described. Formalinized suspensions of various *Shigella* cultures were prepared and tested with native 2-3-476 antiserum (table 1) in dilutions of 1:20 through 1:640. Suspensions of the following cultures were tested: *S. paradyseuterae*, Flexner I, II (two cultures), III, IV, V, and VI; *S. paradyseuterae*, Boyd I, II, III, IV, V, and D.19; culture 2-193, *S. sonnei* (two cultures), *S. alkalascens*, *S. madampensis* (Army Medical School, 43-M-4), and culture 10-888; *Shigella* sp., Sachs Q771, Q1167, Q1030, Q902, A.12, and J.P.6; *S. ambigua*, *S. dysenteriae*, and a number of unclassified cultures.

With four exceptions, these suspensions were unaffected by antiserum 2-3-476. Culture 2-193, 10-888, and *S. sonnei* (Kasauli) agglutinated weakly (1+) in the 1:20 dilution. One *S. sonnei* culture (2-5-750) was agglutinated in the 1:80 dilution. Antiserum 2-3-476 was absorbed with the 2-7-750 *S. sonnei* culture. The factor causing weak agglutination was removed, but the titer of the 2-3-476 antiserum for the homologous culture was not altered.

(6) Although *S. alkalascens* and *S. paradyseuterae*, Boyd V (P.143), cultures did not agglutinate in 2-3-476 antiserum and cultures of the Lavington I type were not agglutinated by *S. alkalascens* or Boyd V antisera, the possibility of the presence of "hidden" antigens in these cultures which might be related was investigated.

S. alkalascens antiserum (1-2) adsorbed with culture 2-3-476 and tested with the homologous *S. alkalascens* (1-2) and 2-3-476 cultures was found to be unaltered by this treatment. Likewise, adsorption of 2-3-476 antiserum with a culture of *S. alkalascens* did not affect the titer for the homologous culture.

Antiserum 2-3-476 was treated with a Boyd V (P.143) culture (8-770). No reduction of titer was noted when the adsorbed antiserum was tested with culture 2-3-476. Nor did adsorption of Boyd V antiserum with culture 2-3-476 result in any alteration of its titer for the Boyd V culture.

(7) Slide agglutinative tests were made with heated (100 C, 30 minutes), unheated, and alcohol-treated suspensions of cultures 2-3-476, 8944, 2-2-4, and Lavington I. Unadsorbed antisera were used in dilutions of 1:5 or 1:10. The 41 antisera employed in the three dilution tube-agglutinative tests, together with *Shigella* sp., Sachs A.12, two polyvalent Flexner antisera, and five Lavington I antisera (table 1) were used in the slide tests.

The unheated, heated, and alcohol-treated suspensions of the four Lavington I type cultures were rapidly and completely agglutinated by all antisera of the Lavington I type at 1:10 dilution. Doubtful, delayed results were obtained in *S. sonnei* (S) and 10-888 antisera, dilution 1:5. The alcohol-treated suspension of culture 8944 and the heated suspension of the Lavington I culture reacted slightly with *S. sonnei* (R) serum at 1:5. Tests with all the other antisera were uniformly negative with the three kinds of suspension.

Formalinized suspensions of cultures 2-3-417, 2-3-1028, and 2-3-1124, and heated suspensions of the last two, were tested in 19 "*Shigella* diagnostic serums" (Lederle) with negative results in all instances.

(8) Serological studies on the possible relationship of the Lavington I serotype to *Salmonella* were made by Captain D. W. Bruner. Eighteen of the cultures listed in table 1 (including 8944, 6900, and Lavington I) were given to Bruner, who prepared alcohol-treated antigens from them in the manner in which *Salmonella* O antigens are prepared (Edwards and Bruner, 1942). The 18 antigens were tested in various antisera containing agglutinins for all the described O antigens of the *Salmonella* group. The antisera were employed in the dilutions used in diagnostic work. No relationship was detected between the antigens of the 18 cultures and the *Salmonella* somatic antigens.

SUMMARY AND DISCUSSION

Biochemical and serological studies were made with a group of 34 cultures recovered from cases of bacillary dysentery and from carriers in North Africa, England, France, and Italy. The purpose of the investigation was to determine whether or not the cultures isolated in the different localities were the same and to determine what, if any, relationship the cultures might have to known *Shigella* and *Salmonella* strains.

The biochemical studies clearly indicate that the microorganisms belong to the genus *Shigella*. The cultures compose a biochemical type made up of nonmotile, gram-negative rods, which are anaerogenic and do not produce acetylmethylcarbinol, utilize citrate, or hydrolyze urea. Lactose is not fermented and hydrogen sulfide is not formed. The criteria for entrance into the genus as listed in *Bergey's Manual* (1939) are therefore fulfilled. Further, the more limiting definition of the genus *Shigella* given by Borman, Stuart, and Wheeler (1944) would also include them except that the question of growth and of acid production at 45 C is unanswered, and the cultures have not been tested on bismuth sulfite agar.

The present group of cultures is remarkably uniform in carbohydrate utilization, the only irregularities being the delayed fermentation of maltose and the variable results with sorbitol. Fermentation of mannitol and lack of acid production from lactose establishes the type as a *Shigella paradysenteriae*. The biochemical activities of the type are identical with those of *S. paradysenteriae*, Boyd V (P.143), as listed by Wheeler (1944). Two stock cultures of the latter type which were available to us differed, however, in that they failed to ferment maltose. These two cultures of Boyd V (P.143) reduced trimethylamine oxide.

Failure to produce acid from dulcitol and rhamnose differentiates the type from typical *S. alkaescens*.

The results of the serological studies indicate that the microorganism isolated by Stock in Casablanca is identical with those recovered by Taylor in England, by Heller in France, and by the writer in Italy. Four antisera prepared with cultures isolated in these localities were adsorbed in all possible combinations. The resulting 64 tests with 16 adsorbed antiserum samples were negative, indicating that agglutinins were removed in all cases. This finding confirms and extends the work of Francis (1945), who found that cultures isolated in England were identical to those recovered in France, as shown by reciprocal adsorptive tests.

The antigenic pattern of the various cultures studied is similar, as is indicated by the fact that each one of 24 cultures is capable of removing the homologous agglutinins from as many aliquots of an antiserum. Cultures isolated since these tests were made were tested with several of these adsorbed (heterologous) antisera by slide tests, and the results were negative in all instances.

No significant relationship between the Lavington I serotype and the other *Shigella* types was revealed when suspensions of the former were tested for agglutination in some 41 antisera prepared against *Shigella* types. Nor did any important relationship become apparent when cultures of various *Shigella* types were tested in an antiserum made with a culture of the Lavington I serotype. One *S. sonnei* culture agglutinated slightly in the 1:80 dilution of 2-3-476 antiserum. This is not interpreted as being significant, however, because the titer of this antiserum was not reduced when it was adsorbed with the *S. sonnei* culture.

The possibility of a relationship of deeper antigens of the cultures was investigated to the extent that "blind" reciprocal adsorptive tests were made with *S. alkaescens*, *S. paradyseuterieae*, Boyd V (P.143), and 2-3-476 cultures, and antisera were prepared against them. No relationship between the Lavington I serotype and the *S. alkaescens* or Boyd V was detected by this means. The possibility remains, however, that such a "hidden" relationship may exist between the Lavington I cultures and some of the other *Shigella* serotypes.

The presence of a heat-labile factor which inhibits agglutination of certain *Shigella* cultures has been mentioned by several investigators (e. g., Stuart, 1944), especially in connection with *S. alkaescens*. Cultures of *S. dispar*, *S. sonnei*, *S. paradyseuterieae* Boyd IV (P.274), *Shigella* sp., Sachs Q1030, and *S. ambigua*, as well as *S. alkaescens*, were encountered in Italy that agglutinated poorly or not at all until after the suspensions were heated. With the Lavington I serotype, however, no such factor was detected, which fact might be responsible for the lack of agglutination in one or another of the various *Shigella* antisera. Heated and alcohol-treated suspensions of several cultures were tested by slide tests in all of our *Shigella* antisera and in commercially prepared antisera with negative results. Such treatment of the suspensions did not affect their agglutinability in Lavington I antisera.

No serological relation was found between the Lavington I serotype and the

Salmonella. These tests were made in connection with an investigation of *Salmonella-Shigella* antigenic relationships that is being carried on by Bruner and the writer.

Thus, the serological studies on the present group of 34 cultures would indicate that they comprise a homogeneous serotype bearing no significant antigenic relation to the other *Shigella* types with which they were compared.

Stock (1945) reports that the outbreak in Casablanca in 1943 in which he isolated 214 cultures of this *S. paradysenteriae* serotype was severe. The cases had temperatures of 103 to 104 F on the first day, and pus and gross blood were present in the stools on the first and second days of illness. By the third day the patients were improved, and after a period of recuperation they were returned to duty.

The cultures isolated by Taylor in England were recovered from 20 cases of bacillary dysentery (Francis, 1945), and those from France occurred in "sporadic outbreaks" (Heller, 1945). Of the 28 cultures isolated in Italy (table 1), 13 were recovered from American soldiers who had bacillary dysentery with typical symptoms and temperatures ranging from 99 to 105 F. The average period of acute illness was 3 days, after which the patients became asymptomatic. One of these cases is complicated by the fact that trophozoites of *Entamoeba histolytica* were reported present in the feces. Two of our cultures were isolated from the same Italian civilian. This individual was ill with dysentery at the time the first culture was isolated. The second was recovered about 3 weeks later, at which time the man was asymptomatic. A blood specimen obtained at the time of the second culture agglutinated the microorganism isolated from the same individual in a dilution of 1:80. Twelve of the cultures were isolated from Italian civilians in the course of routine food handler examinations. In two of these a history was obtained of their having had "diarrhea" during the previous 6 months. We were unable to learn whether or not the other civilians had had dysentery recently. One culture was isolated from a German prisoner of war serving as a food handler; no further information was obtainable in this instance.

It was not possible to obtain enough information about the present group of cultures to furnish all the criteria required to establish the serotype as a pathogen. A similar situation has existed with most of the other *Shigella* strains when they were first reported. The writer feels certain that in time the Lavington I serotype will be accepted, for there is no doubt in his mind as to the pathogenic propensities of the microorganisms.

In regard to the nomenclature of the type, we would suggest that the designation used by the English investigators be retained and the microorganism referred to as *Shigella paradysenteriae*, Lavington I, until such time as taxonomists assign it a number and place it among the other *S. paradysenteriae* serotypes. Heller (1945) has suggested the specific name *Shigella etousa*, but we are of the opinion that a specific name is not warranted or advisable.

CONCLUSIONS

Biochemical and serological studies are described which show that the group of 34 cultures reported upon compose a *Shigella paradysenteriae* type which is

serologically homogeneous and serologically unrelated in any significant way to any other *Shigella* type with which it is compared.

The type is very similar to *S. paradysenteriae*, Boyd V (P.143), in its biochemical activities. Trimethylamine oxide is reduced.

The work indicates that microorganisms of this type isolated in North Africa, England, France, and Italy by several investigators are biochemically and serologically identical.

This serotype is referred to as *S. paradysenteriae*, Lavington I, and it is suggested that this designation be retained until such time as it can be given an appropriate number.

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THE EFFECT OF SULFONAMIDES ON THE ACTION OF PENICILLIN¹

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The remarkable activity of penicillin against various microorganisms has attracted great interest during the past four years. Most of the gram-positive organisms are susceptible to the action of penicillin. Marked differences in susceptibility, however, are to be found not only in organisms of different species but in those within the same species. It has been suggested, therefore, that in some circumstances the combination of other antibacterial agents with penicillin may be advisable when full bacteriostatic or bactericidal action is desired. As the sulfonamides are among the best bacteriostatic agents available, the possible synergistic action of the sulfonamides together with penicillin is of interest.

Ungar (1943) was the first to report the combined use of sulfapyridine with penicillin. He concluded that both *para*-aminobenzoic acid and sulfapyridine act synergistically with penicillin *in vitro* and *in vivo*. Similar results were reported by Bigger (1944), by Soo-Hoo and Schnitzer (1944), by T'ung (1944), and by Kirby (1944). However, Hobby and Dawson (1944a, 1944b) reported that although they could show no evidence of a synergistic action between penicillin and sulfadiazine or sulfapyridine, under the experimental conditions used, they observed a decrease in the rate of growth of the organisms in the presence of sulfapyridine or sulfadiazine and a corresponding decrease in the bactericidal rate of penicillin. This observation was confirmed recently by Garrod (1944), who measured the velocity of disinfection by penicillin and found that it was reduced by about one-half in the presence of a bacteriostatic concentration of sulfathiazole.

In a recent communication Hobby and Dawson (1945) reported briefly experiments showing that under certain conditions sulfadiazine and penicillin in combination will exert a greater bacteriostatic effect than either sulfadiazine or penicillin alone.

EXPERIMENTAL

In the present report experiments illustrating the *in vitro* effect of combined penicillin and sulfadiazine are described in detail.

Effect of Penicillin and Sulfadiazine on Growth of Group A Hemolytic Streptococcus

Cultures of group A *Streptococcus* (strain C₂₀₃Mv) were diluted serially from 10⁻¹ to 10⁻⁷ (1) in broth, (2) in broth containing 8 mg per cent sulfadiazine,

¹ A part of this work was presented before the New York and New Jersey branches of the Society of American Bacteriologists, December, 1944.

(3) in broth containing 0.005 units of penicillin per ml, and (4) in broth containing both 8 mg per cent sulfadiazine and 0.005 units of penicillin per ml. Incubation of each series was at 37 C for 24 hours. The effect of each medium upon the growth of the organism was determined by the presence of turbidity at the end of the incubation period.

Under these experimental conditions sulfadiazine alone caused an inhibition of growth in dilutions greater than 10^{-3} . Penicillin alone and likewise penicillin and sulfadiazine combined inhibited growth in dilutions greater than 10^{-2} . Penicillin and sulfadiazine in combination therefore were unable to inhibit a larger number of organisms than penicillin alone (table 1).

The Effect of Sulfadiazine on the Action of Penicillin in Vitro

Further experiments were conducted by the serial dilution method to determine the minimum amount of penicillin which would completely inhibit growth in plain broth and in broth containing 8 mg per cent sulfadiazine. A strain highly

TABLE 1
Effect of penicillin and sulfadiazine on growth of hemolytic Streptococcus (group A)

Medium*	DILUTION OF ORGANISMS						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Broth control.....	++++	++++	++++	++++	++++	++	++
Sulfadiazine 8 mg%.....	++++	++++	+++	±	±	—	—
Sulfadiazine 8 mg% + penicillin 0.005 U/ml.....	++++	++++	—	—	—	—	—
Penicillin 0.005 U/ml.....	++++	++++	—	—	—	—	—

Readings: —, +, ++, +++, +++++ indicate amount of turbidity.

* Basic medium: beef infusion broth.

sensitive to sulfadiazine, group A *Streptococcus* (C₂₀₈Mv), and two strains highly resistant to sulfadiazine, *Staphylococcus aureus* (H) and a recently isolated strain of viridans *Streptococcus* (Mor.), were used. Incubation was at 37 C for 24 hours, and the amount of growth as evidenced by turbidity was observed.

In the case of the sulfadiazine-sensitive strain of hemolytic *Streptococcus* the amount of penicillin necessary to inhibit growth was decreased from 0.015 units per ml to 0.008 units per ml when sulfadiazine was present. Sulfadiazine in no way affected the amount of penicillin necessary to inhibit the growth of the other two strains, both of which were highly resistant to the action of the sulfonamides. It was apparent that sulfadiazine may at times decrease the amount of penicillin necessary to produce bacteriostasis against a constant number of organisms. Any effect which sulfadiazine exerts, however, may be dependent at least in part on the sensitivity of the organism to this drug (table 2).

It has been shown previously that the sulfonamides exert a bacteriostatic action only on small numbers of organisms and after a 5- to 7-hour lag period during which the drug is in contact with the cell (Chandler and Janeway, 1939).

Large amounts of penicillin are known to exert a rapid bactericidal action even against large numbers of organisms, whereas small amounts of penicillin exert only a mild bacteriostatic effect (Hobby and Dawson, 1944; Hobby *et al.*, 1942). It seemed likely, therefore, that the range in which sulfadiazine might increase the bacteriostatic action of penicillin would be narrow. Subsequent experiments therefore were undertaken to determine this range.

The In Vitro Effects of Varying Concentrations of Penicillin Combined with Sulfadiazine

Hemolytic *Streptococcus* (strain C₂₀₃Mv) and *Staphylococcus aureus* (strain H) were used. The minimum amount of penicillin necessary to inhibit growth of these organisms in sterile broth was determined by the multiple serial dilution method. In the case of group A *Streptococcus* (strain C₂₀₃Mv) 0.015 to 0.03 units usually were adequate to inhibit 100,000 to 4,000,000 organisms. Occasionally larger amounts were necessary for complete inhibition. Smaller numbers

TABLE 2
Effect of sulfadiazine on action of penicillin in vitro

STRAIN	SENSITIVITY TO SULFADIAZINE	SENSITIVITY TO PENICILLIN	
		In broth	In broth containing sulfadiazine
		<i>U per ml</i>	<i>U per ml</i>
Group A <i>Streptococcus</i> (C ₂₀₃ Mv)	+++	0.015	0.008
<i>Staphylococcus aureus</i> (strain H).....	—	0.03	0.03
Viridans <i>Streptococcus</i> (Mor.).....	—	0.18	0.18

Concentration of sulfadiazine used: 8 mg per cent.

* The sensitivity of organisms to penicillin was measured by the minimum number of units that would completely inhibit the growth of 3 to 4 million organisms.

of organisms (300 to 400) were inhibited by approximately 0.002 units. In the case of *Staphylococcus aureus* (strain H) 0.03 to 0.05 units were required to inhibit 100,000 to 4,000,000 organisms, whereas 0.008 to 0.01 unit was adequate to inhibit 300 to 500 organisms. The sensitivity of these strains to sulfadiazine was also determined. It was found that 8 mg per cent sulfadiazine was effective against group A *Streptococcus* (strain C₂₀₃Mv) in a concentration not greater than 30,000 to 40,000 organisms per ml. This amount of sulfadiazine was completely ineffective against even small numbers of *Staphylococcus aureus* (strain H). With these results as a basis for estimating the amount of bacteriostasis that would result under any given set of conditions, the following experiments were conducted.

High concentrations of sulfadiazine-sensitive group A Streptococcus (strain C₂₀₃Mv). Four tubes containing 10 ml of sterile broth and 4 containing 10 ml of broth to which had been added 8 mg per cent sulfadiazine were set up. Penicillin was added to 1 tube from each series in an amount sufficient to give a final concentration of 0.05 unit per ml, a concentration probably in excess of that neces-

sary for complete inhibition. Penicillin was also added to tubes from each series in an amount slightly less than that necessary for complete inhibition (0.01 unit per ml) and likewise in an amount small enough to produce only slight bacteriostasis (0.005 units per ml). One tube from each series was held as a control. One ml of a 10^{-1} dilution of group A *Streptococcus* was added to each. The final concentration of organisms was approximately equivalent to 10^{-2} ; that of sulfadiazine was equivalent to 7.2 mg per cent. All tubes were incubated at 37 C. The number of organisms per ml was determined by plate counts at various intervals.

The initial number of organisms was high. Rapid multiplication took place in sterile broth. With this heavy inoculum there was little or no bacteriostasis in the presence of 7.2 mg per cent sulfadiazine alone. Penicillin alone, in a concentration of 0.005 units per ml, at times caused a more marked bacteriostasis. There was always, however, an actual increase in the concentration of organisms. Sulfadiazine in combination with 0.005 units of penicillin per ml produced no significantly greater bacteriostatic effect than did this concentration of penicillin alone. In the presence of a higher concentration (0.01 unit per ml) of penicillin alone there was a short lag phase during which no multiplication took place. Following this lag phase penicillin exerted either a definite bactericidal action, which destroyed many of the organisms present at the end of the lag phase, or a marked bacteriostatic effect, which prevented rapid growth of the organisms. After a period of 8 to 10 hours multiplication took place rapidly in the presence of 0.01 unit of penicillin per ml. The bacteriostatic or bactericidal action continued even after 10 hours when 7.2 mg per cent sulfadiazine was combined with 0.01 unit of penicillin per ml. A rapid drop in the bacterial count occurred in the presence of 0.05 units of penicillin per ml either alone or with sulfadiazine added. Representative results are shown in tables 3 and 4.

In the experiment described above, the initial number of organisms was large. Penicillin in a concentration of 0.005 units per ml produced little bacteriostasis. The concentration of organisms at the end of 5 to 7 hours remained high. Therefore, since sulfadiazine is effective only against small numbers of organisms and only after being in contact with the organism for a period of 5 to 7 hours, sulfadiazine was unable to exert any bacteriostatic action on the organism (Chandler and Janeway, 1939). When penicillin was present in a concentration of 0.01 unit per ml, the number of organisms decreased, within 5 to 7 hours, to a concentration on which sulfadiazine, if present, could act. When sulfadiazine was present in combination with penicillin, the action of sulfadiazine became apparent and the number of organisms decreased until complete sterilization had occurred. The bactericidal action in the presence of an excess of penicillin (0.05 units per ml) was rapid and for the most part occurred during the first 5- to 7-hour period. When sulfadiazine was added, the bactericidal action was not enhanced.

Low concentrations of sulfadiazine-sensitive group A Streptococcus (C₁₃₃Mv). Since the sulfonamides are more effective against small numbers of organisms, experiments were conducted using an initial seeding of smaller numbers of group A *Streptococcus*. Four tubes containing 10 ml of sterile broth and 4 containing

10 ml of broth to which had been added 8 mg per cent sulfadiazine were set up. Penicillin was added to 1 tube from each series in an amount sufficient to give a

TABLE 3
Action of sulfadiazine and penicillin on hemolytic Streptococcus (group A)
(Initial concentration of organisms high)

HOURS	SULFADIAZINE: MG PER CENT							
	0	7.2	0	7.2	0	7.2	0	7.2
	Penicillin: U per ml							
	0	0	0.005	0.005	0.01	0.01	0.05	0.05
	No. of organisms \times 1,000 per ml							
0	3,750	3,750	3,750	3,750	3,750	3,750	3,750	3,750
2	9,200	11,000	1,040	1,392	8,000	4,400	2,700	2,000
4	23,500	18,800	3,120	2,460	870	870	88	845
6	52,500	69,900	7,050	7,680	420	320	33	36
8	160,000	65,500	25,500	12,500	207	85	0.1	2.9
24	73,500	44,000	420	255	7,000	0.1	0	0

Basic medium: beef infusion broth.

TABLE 4
Action of sulfadiazine and penicillin on hemolytic Streptococcus (group A)
(Initial concentration of organisms high)

HOURS	SULFADIAZINE: MG PER CENT							
	0	7.2	0	7.2	0	7.2	0	7.2
	Penicillin: U per ml							
	0	0	0.005	0.005	0.01	0.01	0.05	0.05
	No. of organisms \times 1,000 per ml							
0	116	116	116	116	116	116	116	116
1½	204	220	141	184	89	244	116	120
3	515	1,960	346	950	202	200	49.5	44.3
5	1,320	2,800	610	2,460	389	646	4.8	2.6
10	61,800	31,900	14,850	15,400	938	2,280	1.8	0.5
12		132,000	61,700	48,100	1,700	1,047	0.3	0
13-14	305,000	100,000	43,000			30.7		0
16	195,000	73,000			8,030	3.8	0	
19-20	151,000	90,000	42,000		23,500	1.0	0	0
25	120,000	64,000	42,500	34,500	21,500		0	0
64	44,000	20,500			1,600	0	0	0
72	14,500		4,000	9,000		0	0	0

Basic medium: beef infusion broth.

final concentration of 0.008 units per ml, 0.003 units per ml, and 0.001 unit per ml. One tube from each series was held as a control. One ml of a 10^{-8} dilution of *Streptococcus* ($C_{200}Mv$) was added to each tube. The final concentration of

organisms was approximately 10^{-6} ; that of sulfadiazine was 7.2 mg per cent. All tubes were incubated at 37 C. The number of organisms per ml was determined by plate counts at intervals.

The initial concentration of organisms was low. Rapid multiplication took place in plain broth and in broth containing 0.001 to 0.003 units of penicillin per ml. The bacteriostatic action of sulfadiazine alone in a concentration of 7.2 mg per cent was marked. Bacteriostasis also occurred after 5 to 7 hours' incubation, however, when sulfadiazine was present in combination with 0.001 to 0.003 units of penicillin per ml. The bacteriostatic effect observed probably was due entirely to the sulfadiazine. In the presence of 0.008 units of penicillin per ml, the organisms rapidly decreased in number before the end of the first 5 to 7 hours. When sulfadiazine was added to this amount of penicillin, there was at times a prolonged lag period before the destruction of the organisms. No significant

TABLE 5

Action of sulfadiazine and penicillin on hemolytic Streptococcus (group A)

(Initial concentration of organisms low)

HOURS	SULFADIAZINE: MG PER CENT							
	0	7.2	0	7.2	0	7.2	0	7.2
	Penicillin: U per ml							
	0	0	0.001	0.001	0.003	0.003	0.008	0.008
	No. of organisms \times 1,000 per ml							
0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
7	175	36.5	62	29	11.5	114	0	0.3
30	3,200		8,000	5.7	57,000	0	0	0
48	48,000	2	24,000	0.1	62,000	0	0	0

Basic medium: beef infusion broth.

difference was observed, however, between the ultimate effect of this concentration of penicillin when alone and when combined with sulfadiazine (table 5).

Low concentration of sulfadiazine-resistant Staphylococcus aureus (strain H). Similar experiments were conducted using *Staphylococcus aureus* (strain H), which is resistant to sulfadiazine. Four tubes containing 10 ml of sterile broth and 4 containing 10 ml of broth to which had been added 8 mg per cent sulfadiazine were set up. Penicillin was added to 1 tube from each series in an amount sufficient to give a final concentration of 0.1 unit per ml, an amount in excess of that necessary for inhibition, and in an amount sufficient to give a final concentration of 0.01 unit per ml, a concentration slightly less than the minimum amount necessary for complete inhibition. Penicillin was also added to 1 tube from each series in amounts small enough to give little or no bacteriostasis (0.005 and 0.001 unit per ml). One tube from each series was held as a control. One ml of a 10^{-5} dilution of *Staphylococcus aureus* (H) was added to each. The final concentration of organisms was 10^{-6} ; that of sulfadiazine was 7.2 mg per

cent. All tubes were incubated at 37 C. The number of organisms was determined by plate counts at various intervals.

The initial number of organisms was low. Rapid multiplication took place both in broth and in broth containing 7.2 mg per cent sulfadiazine. Penicillin alone in a concentration of 0.005 or 0.001 unit per ml caused no bacteriostasis, whereas these concentrations of penicillin, in combination with 7.2 mg per cent sulfadiazine, produced slight bacteriostasis for a period of approximately 8 hours. In a concentration of 0.01 unit per ml, penicillin alone produced a more marked bacteriostatic effect. Multiplication took place at a rate much slower than in plain broth. Eventually, however, there was an increase in the rate of multiplication and in the number of organisms present. Sulfadiazine in combination with this amount of penicillin increased and prolonged the bacteriostatic effect

TABLE 6
Action of sulfadiazine and penicillin on Staphylococcus aureus
(Initial concentration of organisms low)

HOURS	SULFADIAZINE: MG PER CENT									
	0	7.2	0	7.2	0	7.2	0	7.2	0	7.2
	Penicillin: U per ml									
	0	0	0.001	0.001	0.005	0.005	0.01	0.01	0.1	0.1
No. of organisms \times 1,000 per ml										
0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5		
8	9,850	1,000	5,450	390	3,900	90	155	1.9		
31	750,000	310,000	450,000	137,000	180,000	115,000	210,000	38		
48	885,000	220,000	461,000	117,000	169,000	115,000	83,000	27,800		
0	0.23	0.23							0.23	0.23
3	8.2	8.1							0.14	0
6	660	49							0	0
9	555,000	1,712							0	0
24	1,535,000	120,000							0	0

on this organism. Definite bactericidal action was observed with 0.1 unit of penicillin per ml, both alone and in the presence of sulfadiazine (table 6).

High concentrations of sulfadiazine-resistant and penicillin-resistant hemolytic Streptococcus (group D). The effect of sulfadiazine in combination with penicillin upon a strain of group D *Streptococcus* which is relatively resistant to both antibacterial agents was next determined. The culture used was a hemolytic serological group D *Streptococcus* (strain E), freshly isolated from a case of sub-acute bacterial endocarditis. Penicillin in a concentration of 3 units per ml exerted a bacteriostatic action against this organism. This bacteriostatic effect, however, was only temporary. No bacteriostasis whatsoever was observed with smaller amounts of penicillin, or with sulfadiazine in amounts up to 100 mg per cent.

Six tubes containing 6.6 ml of sterile broth and 6 tubes containing 6.6 ml of broth to which had been added 3 units of penicillin per ml were set up. Sulfadiazine was added to 1 tube from each set (1) in an amount sufficient to give a final concentration of 2 mg per cent, (2) 4 mg per cent, (3) 6 mg per cent, (4) 8 mg per cent, and (5) 10 mg per cent. One tube from each set was held as a control. Each tube was corrected to a volume of 7.5 ml and was seeded with a dilution of culture sufficient to give a final concentration of 10^{-2} (approximately 4,000,000 organisms per ml). Incubation was at 37 C for 24 hours. Growth was determined by the degree of turbidity at the end of the incubation period. Rapid growth occurred in all tubes. No inhibition whatsoever was observed.

Six tubes containing 6.2 ml of sterile broth and 6 tubes containing 6.2 ml of broth to which had been added 8.0 mg per cent sulfadiazine were set up subsequently. Penicillin was added to 1 tube from each set to give a final concentra-

TABLE 7
Action of sulfadiazine and penicillin on hemolytic Streptococcus (group D)

MEDIUM*		AMOUNT OF GROWTH		
Sulfadiazine	Penicillin	24 hours	48 hours	72 hours
mg per cent	U per ml			
0	0	++++	++++	++++
7.2	0	++++	++++	++++
0	0.5	++++	++++	++++
7.2	0.5	++++	++++	++++
0	1.0	++++	++++	++++
7.2	1.0	+++++	+++++	+++++
0	2.0	++	++	++++
7.2	2.0	++	++	+++
0	3.0	±	++	++++
7.2	3.0	-	+	+
0	4.0	±	++	++++
7.2	4.0	-	-	-

* Basic medium: beef infusion broth.

tion of (1) 0.5 U per ml, (2) 1 U per ml, (3) 2 U per ml, (4) 3 U per ml, and (5) 4 U per ml. The volume of each tube was adjusted to 6.7 ml by the addition of sterile broth. Group D *Streptococcus* (strain E) was added in sufficient quantity to give a final concentration of 10^{-2} . The final concentration of sulfadiazine was 7.2 mg per cent. Incubation was again at 37 C, and the amount of growth as evidenced by turbidity was read at 24, 48, and 72 hours.

Heavy growth took place in plain broth and in broth containing 7.2 mg per cent sulfadiazine. Likewise no bacteriostasis occurred in the presence of 0.5 or 1.0 unit of penicillin per ml or in the presence of sulfadiazine in combination with such a concentration of penicillin. With larger amounts of penicillin (2.0 to 4.0 U per ml) temporary bacteriostasis took place. When sulfadiazine was present in combination with these amounts of penicillin, the bacteriostasis was prolonged. However, only in the presence of 4 units of penicillin per ml combined with 7.2

mg per cent sulfadiazine was the inhibition of growth complete and permanent (table 7).

An organism which is inhibited only temporarily by penicillin and is not inhibited by sulfadiazine can apparently be inhibited permanently by their combined action under certain experimental conditions. Sulfadiazine will not enhance the action of a subminimal amount of penicillin. However, if sufficient penicillin is present to produce partial bacteriostasis before the end of the sulfonamide lag phase, sulfadiazine may enhance the bacteriostatic action of the penicillin even though the organism appears to be sulfadiazine-resistant.

DISCUSSION

The results obtained by the combined use of penicillin and sulfadiazine are dependent on many factors. Among these are (1) the concentration of each bacteriostatic agent, (2) the number of organisms present, (3) the environmental conditions allowing growth of the organism, (4) the degree of susceptibility of the organism to penicillin and to sulfadiazine, and (5) the individual species of organism involved.

In the presence of penicillin in amounts so small as to produce little or no bacteriostatic action, sulfadiazine increases bacteriostasis provided that the organism is sulfadiazine-sensitive and that it is present in small numbers only. In this instance the bacteriostatic effect is predominantly due to sulfadiazine.

In the presence of penicillin in amounts sufficient to produce a definite bacteriostatic or bactericidal effect during the first few hours of incubation but insufficient to yield complete sterilization, sulfadiazine appears to increase bacteriostasis provided the number of organisms present at the end of the sulfadiazine lag period is low. Sensitivity of the organism to sulfadiazine enhances this effect, but a degree of sensitivity sufficient to be demonstrable by the usual means may not be invariably essential.

In the presence of larger amounts of penicillin the bactericidal action of penicillin is rapid during the first few hours of incubation. Complete sterilization may result before 5 to 7 hours. If sterilization is not complete at the end of this time, the presence of sulfadiazine at times may prolong the lag period or even slightly decrease the bactericidal rate provided the organism is sensitive to sulfadiazine and is present in small numbers. The action of penicillin occurs predominantly at the time of cell division. A decrease in the rate of multiplication due to sulfadiazine therefore may decrease the rate at which penicillin acts.

The number of strains studied and the number of experiments conducted necessarily have been limited. All, however, have been repeated on several occasions with the same results. The data accumulated suggest that the combined use of sulfadiazine and penicillin at times may produce greater bacteriostasis than either alone. It has not been proved whether the two agents act independently of each other or whether one increases the activity of the other. The fact that sulfadiazine appears to increase the activity of penicillin against one strain resistant to penicillin alone suggests the possibility that penicillin may alter the bacterial cell so as to increase sensitivity to sulfadiazine.

The difficulty involved in evaluating the conditions present in the body is obvious. With the average therapeutic dose now used, penicillin is generally present in the blood stream in amounts well in excess of that necessary to inhibit a sensitive organism, but less than might be necessary, however, in the case of more resistant organisms. Then, too, the amount of penicillin reaching massive infections in localized areas may be minimal. It is probably impossible to determine in advance whether or not sulfadiazine will enhance the bacteriostatic action of penicillin in human infection.

CONCLUSIONS

The combination of penicillin and sulfadiazine produces, at times, a greater bacteriostatic effect *in vitro* than the same concentration of sulfadiazine or penicillin alone. However, specific experimental conditions are necessary to produce this effect.

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INFLUENCE OF INCUBATION TEMPERATURES ON DIFFERENTIAL TESTS OF COLIFORM BACTERIA

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In the bacteriological testing of rural raw water supplies the laboratory is having to face, constantly, the problem of evaluating the sanitary significance of the atypical coliform bacteria so often recovered from positive tests. For several years the author and associates have carried on studies of these bacteria as a corollary to the water-testing service that is maintained for the rural public of the state. A review of some of these studies has been published (Fuller, 1944).

The study here reported is another in the series. The object was to determine the reactions of coliform bacteria, recovered from raw water, to differential tests at incubation temperatures ranging from the conventional 37 C to the Eijkman temperature of 46 C. The hope was that the information gained would be of help in determining the relation of atypical or intermediate coliform bacteria to fecal or nonfecal types.

The Eijkman (1904) test has not had popular support in recent years among sanitary bacteriologists in this country. Its use in modified form, however, has been recommended by Perry and Hajna (1933, 1935) for detecting coliform bacteria, particularly *Escherichia coli*, in shellfish. Their experience prompted some work in this laboratory by France (1938) and Levine (1940). France observed that certain coliform strains were able to produce acid, but not gas, in lactose broth at 46 C, and Levine found that certain strains produced gas at higher incubation temperatures in the modified Eijkman medium of Perry and Hajna (1933) than in the lactose broth of the Standard Methods. These experiences suggested the study which is the subject of this paper.

EXPERIMENTAL

The tests employed in this study were gas production in lactose broth, reaction on Endo's agar, and "the Imvic tests" (Parr, 1936), i.e., indole, methyl red, Voges-Proskauer, and sodium citrate tests. All media were made and tests were conducted as directed in the *Standard Methods of Water Analysis* (8th ed., 1936) with the exception that the Gore (1921) plug test was employed for indole. Endo's medium was streaked at 24 and 48 hours from lactose broth cultures grown at the several temperatures employed. The streaked plates were incubated at 37 C and observed at 24 and 48 hours.

After some preliminary experiments with laboratory cultures, a series of 125 freshly isolated coliform cultures were taken for study. They were isolated from positive tests of samples sent to the laboratory for routine testing and were purified by the method of Ruchhoft *et al.* (1931).

¹ Contribution No. 575.

The selection of incubation temperatures was based largely on usages, as follows: 37 C is that commonly employed, and 46 C is the Eijkman temperature. According to McCrady (1939), 44 C is recommended in the British Standard Methods for the bacteriological examination of water with MacConkey's broth, and references in the literature indicate that this temperature is favored for the selective cultivation of *E. coli* and closely related bacteria (Clegg, 1941; Sherwood and Clegg, 1942; Raven, Pedan, and Wright, 1940). Incubation at 40 C was added to try the effect of a temperature midway between 37 and 44 C. Thus, the incubation temperatures chosen for this study were 37, 40, 44, and 46 C.

Several methods of incubation were investigated. First, an electrically heated and thermostatically controlled water bath was employed. Then, duplicate sets of cultures were incubated, one in the water bath and the other in a water-jacketed, electrically heated laboratory incubator, with little to choose between them. The third method, and the one selected for use, was incubation in a forced-draft, electrically heated incubator, which proved, by experiment, to be superior to either of the other methods mentioned for maintaining constant temperatures. Control was possible within a range of about 0.5 C. The temperatures mentioned in the text and tables were maximum in each instance.

The media were heated to the desired temperature before they were used, and inoculated tubes were returned promptly to the incubator. This proved to be particularly important at 40 C and above. Two successive sets of tests were made, with some repeating of tests in which the results were in doubt. Then, 6 months later, the experiments were repeated with no significant differences in results.

The results of the tests are shown in tables 1 through 5. On the basis of their reactions at 37 C, the cultures were grouped in what might be termed a sanitary classification, as follows:

	Indole	M.R.	V.P.	No-citrate
<i>Escherichia coli</i>	+	+	—	—
<i>Citrobacter</i>	+	+	—	+
<i>Aerobacter aerogenes</i>	—	—	+	+

Since indole production is likely to be variable among coliform strains, three additional groupings were made: indole-negative *E. coli*, indole-negative *Citrobacter*, and indole-positive *A. aerogenes*. The intermediates, those strains that did not fit into these six groups, were placed in three additional groups on the basis of their Imvic reactions, as shown in the tables. The term "*Citrobacter*" (Werkman and Gillen, 1932) has not been admitted into biological classifications, but it is useful for description in sanitary studies.

Gas production in lactose broth. Reference to table 1 shows that all of the *E. coli* strains produced gas through 44 C, and all but 4 did so at 46 C. Few of the other strains (7 of 79) produced gas at 46 C; and of the 44 strains other than *E. coli* that produced gas at 44 C, it will be noted that 28 were *A. aerogenes*. Four of the latter were among the 7 strains, other than *E. coli*, that produced gas at 46 C.

From the data in the table it may be observed that, of the strains studied, *E. coli* was outstanding in its ability to produce gas at the Eijkman temperature. This finding agrees with Eijkman's claim and with the claims of supporters of the principle of the Eijkman test.

It may be observed also that, although strains such as the indole-negative *E. coli*, *Citrobacter*, and indole-negative *Citrobacter* may appear to be related to *E. coli* so far as some of their reactions are concerned, their weakness in gas production at 46 C puts them in the company of strains other than *E. coli*. Thus, *E. coli* stands out in gas-producing capacity at 46 C, and if this fact is accepted as a criterion of fecal origin, only *E. coli* strains would be considered indicative of fecal pollution.

Incubation at 44 C did not prove to be selective for detecting *E. coli* on the basis of gas production, since a number of the other strains tested, and notably

TABLE 1
Influence of incubation temperature on gas production in lactose broth

GROUP	NO. OF CULTURES	TEMPERATURE, C				
		37	40	44	46	No visible growth, 46
<i>E. coli</i>	46	46	46	46	42	0
<i>E. coli</i> , indole-negative	11	11	9	7	0	0
<i>Citrobacter</i>	5	5	4	3	2	0
<i>Citrobacter</i> , indole-negative	12	12	9	1	0	8
<i>A. aerogenes</i>	36	36	32	28	4	0
<i>A. aerogenes</i> , indole-positive	4	4	4	3	1	0
Intermediates, Imvic - + + +	5	5	3	1	0	0
Intermediates, Imvic - - + -	4	4	3	1	0	1
Intermediates, Imvic - + + -	2	2	2	0	0	2
Totals	125	125	112	90	49	11

Note: Figures in columns under temperatures indicate positives.

28 (77 per cent) of the *A. aerogenes* strains, produced gas at that temperature. The advocates of 44 C incubation cited earlier in this report employed MacConkey's broth, so to that extent this study differed from theirs; but under the conditions of the present experiment, incubation at 44 C for the detection of *E. coli* is not indicated.

A further observation may be made: of the 11 strains that gave no visible growth at 46 C, there were no *A. aerogenes* or indole-positive *A. aerogenes*, both of which are considered to be nonfecal coliform types. This result indicates the ability of *A. aerogenes* to survive and multiply at the Eijkman temperature.

Endo plates. Endo plates were streaked from lactose broth cultures that had been incubated at the several temperatures employed. The plates were incubated at 37 C. Tabular presentation is dispensed with since *E. coli* and indole-negative *E. coli* almost all gave growth typical of *E. coli* throughout the temperature range, and all the other groups, including *A. aerogenes*, gave variable

results at temperatures above 37 C. This does not indicate a close relationship between *Citrobacter* and either indole-positive or indole-negative *E. coli*.

Indole test. Although it was not expected that strains that were indole-negative at 37 C would produce indole at higher temperatures, the tests were made at all temperatures for the sake of assurance. The results conformed to expectation on that point.

It will be observed from table 2 that all but two of the *E. coli* strains produced indole through 46 C, and of the two, one was negative at 40 C and the other at 44 C. The numbers of indole-positive *Citrobacter* and *A. aerogenes* strains were not large, but the results indicated a definite tendency for them to become indole-negative before 46 C was reached. This agreed with the evidence from the lactose fermentation in suggesting that these organisms are not so closely related to *E. coli* as tests at 37 C would indicate.

TABLE 2
Influence of incubation temperature on the indole test

GROUP	NO. OF CULTURES	TEMPERATURE, C			
		37	40	44	46
<i>E. coli</i>	46	46	45	44	44
<i>E. coli</i> , indole-negative....	11	0	0	0	0
<i>Citrobacter</i>	5	5	4	3	2
<i>Citrobacter</i> , indole-negative	12	0	0	0	0
<i>A. aerogenes</i>	36	0	0	0	0
<i>A. aerogenes</i> , indole-positive..	4	4	4	3	1
Intermediates, Imvic - + + +	5	0	0	0	0
Intermediates, Imvic - - + -	4	0	0	0	0
Intermediates, Imvic - + + -	2	0	0	0	0
Totals.....	125	55	53	50	47

Note: Figures in columns under temperatures indicate positives.

Methyl red test. In some preliminary experimental work some coliform strains giving methyl-red-negative tests at 37 C appeared to become positive at higher temperatures, indicating that the higher temperatures did not prevent acid production but apparently did prevent the alkali reversion that is characteristic of *A. aerogenes* and some other methyl-red-negative strains. Consequently, it was decided to test all of the strains employed in this study for assurance. A few of the methyl-red-negative strains (at 37 C) from this series did give some positive tests at higher temperatures, but duplicates did not agree and retesting failed to confirm the changes in reaction. Consequently, table 3 shows no methyl-red-positive results among cultures that were negative at 37 C. Strains positive at 37 C showed consistent results through 44 C, but at 46 C there was a marked drop in positives among all of the strains employed, *E. coli* included.

The result of the methyl red test shows evidence of relationship among the *E.*

coli and *Citrobacter* strains, including those that are indole-negative, whereas the lactose broth fermentation and indole tests had indicated otherwise. Acid production from carbohydrates, on which the methyl red test is based, is considered by many authorities to be the most significant single criterion for the separation

TABLE 3
Influence of incubation temperature on the methyl red test

GROUP	NO. OF CULTURES	TEMPERATURE, C			
		37	40	44	46
<i>E. coli</i>	46	46	46	46	11
<i>E. coli</i> , indole-negative..	11	11	11	10	2
<i>Citrobacter</i>	5	5	5	4	1
<i>Citrobacter</i> , indole-negative. .	12	12	12	11	1
<i>A. aerogenes</i>	36	0	0	0	0
<i>A. aerogenes</i> , indole-positive.....	4	0	0	0	0
Intermediates, Imvic - + + +	5	5	4	3	0
Intermediates, Imvic - - + -	4	0	0	0	0
Intermediates, Imvic - + + -	2	2	2	0	0
Totals.....	125	81	80	74	15

Note: Figures in columns under temperatures indicate positives.

TABLE 4
Influence of incubation temperature on the Voges-Proskauer test

GROUP	NO. OF CULTURES	TEMPERATURE, C			
		37	40	44	46
<i>E. coli</i>	46	0	0	0	0
<i>E. coli</i> , indole-negative.....	11	0	0	0	0
<i>Citrobacter</i>	5	0	0	0	0
<i>Citrobacter</i> , indole-negative.....	12	0	0	0	0
<i>A. aerogenes</i>	36	36	25	3	0
<i>A. aerogenes</i> , indole-positive	4	4	2	1	0
Intermediates, Imvic - + + +	5	5	1	0	0
Intermediates, Imvic - - + -	4	4	1	0	0
Intermediates, Imvic - + + -	2	2	2	0	0
Totals.....	125	51	31	4	0

Note: Figures in columns under temperatures indicate positives.

of fecal from nonfecal coliform bacteria. If that position is accepted, the relationship between *E. coli* and *Citrobacter*, whether they are indole-positive or indole-negative, is supported by the methyl red results through 44 C.

Voges-Proskauer test. Table 4 gives the results of this test. All strains were tested, even though it was anticipated, and proved to be true, that strains that

were V.-P.-negative at 37 C would remain negative at higher temperatures. Although the results of this test do not have much bearing on the general objective of this investigation, it may be observed from the table that V.-P.-positiveness decreased quickly as temperatures were raised, a drop of 40 per cent between 37 and 40 C.

Sodium citrate test. As with the V.-P. test, all strains were tested at all temperatures, even though they were negative at 37 C. The data in table 5 show that the elevation of incubation temperatures resulted in a rapid decrease in the number of citrate-positive tests. Of the 68 strains which were positive at 37 C, 53 remained positive at 40 C, a drop of about 23 per cent, and the decrease in positive strains of *A. aerogenes* was greater still (about 33 per cent). At 44 C the number of positives was negligible, and there were none at 46 C.

TABLE 5
Influence of incubation temperature on the sodium citrate test

GROUP	NO. OF CULTURES	TEMPERATURE, C			
		37	40	44	46
<i>E. coli</i>	46	0	0	0	0
<i>E. coli</i> , indole-negative.....	11	0	0	0	0
<i>Citrobacter</i>	5	5	5	0	0
<i>Citrobacter</i> , indole-negative.....	12	12	11	0	0
<i>A. aerogenes</i>	36	36	25	3	0
<i>A. aerogenes</i> , indole-positive.....	4	4	4	1	0
Intermediates, Imvic - + + +.....	5	5	5	0	0
Intermediates, Imvic - - + -.....	4	4	1	0	0
Intermediates, Imvic - + + -.....	2	2	2	0	0
Totals.....	125	68	53	4	0

Note: Figures in columns under temperatures indicate positives.

COMMENT

Consideration of the data in the tables, makes it apparent that three of the tests might be regarded as significant in indicating the relationship of the coliform bacteria to either *E. coli* or *A. aerogenes*. Two of these tests were the production of gas from lactose broth and the indole test. In the former, *E. coli*, not including the indole-negative *E. coli*, was outstanding among the strains studied in being able to produce gas at 46 C, an observation that agrees with Eijkman's original claim. Since the indole test is admitted to be variable among coliform bacteria, not too much should be expected of it, but the data in the tables show that no relationship was demonstrated between indole-positiveness and ability to produce gas at incubating temperatures above 37 C. The third of the tests, the methyl red test, gave results that suggest a definite relationship between *E. coli*, on the one hand, and indole-negative *E. coli*, *Citrobacter*, and indole-negative *Citrobacter*. This relationship persisted through 44 C, but at

46 C none of the cultures employed, including *E. coli*, gave a sufficiently large number of positive methyl red tests to suggest the use of that temperature for the test.

The methyl red test has been strongly supported by many investigators as the most valuable single test for differentiating *E. coli* and closely related strains from other coliform bacteria. As a result of the experience in these studies, it is suggested that running the methyl red test at 44 C might prove to be a useful technique for evaluating the sanitary significance of coliform bacteria isolated from raw waters.

SUMMARY

Coliform cultures isolated from rural water supplies were studied to determine the effect of incubation temperatures (37, 40, 44, and 46 C) on their reactions to differential tests.

Only *E. coli* was able to produce gas effectively in lactose broth at 46 C. This test was selective only for indole-positive *E. coli* as contrasted with the other strains studied.

Endo's medium plates were streaked from lactose broth cultures incubated at the several temperatures. The plates, incubated at 37 C, showed typical results for *E. coli* throughout the temperature range employed. Results for other strains were variable and lacked definition.

The results of the methyl red test at 44 C indicated a definite relationship of all *E. coli* and *Citrobacter* strains, including those that were indole-negative. The number of positive tests declined substantially at 46 C.

The Voges-Proskauer and sodium citrate tests gave no significant information at temperatures above 37 C.

The results of the study suggest that the methyl red test at 44 C may be useful as a confirmation test in evaluating the sanitary significance of atypical or intermediate strains of coliform bacteria isolated from raw water.

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MICROBIOLOGICAL ASPECTS OF PENICILLIN

IV. PRODUCTION OF PENICILLIN IN SUBMERGED CULTURES OF *PENICILLIUM NOTATUM*¹

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Available information on the production of penicillin deals exclusively with surface cultures of *Penicillium notatum* in shallow layers of medium (Abraham *et al.*, 1941; Challinor, 1942; Clutterbuck *et al.*, 1932; Fleming, 1929, 1932; Hobby *et al.*, 1942; Kocholaty, 1942; Reid, 1935). The need for large amounts of this therapeutic agent led to investigation of submerged cultures of *P. notatum* as a practicable process for large-scale production of penicillin. This paper shows that substantial amounts of penicillin can be produced in submerged cultures of *P. notatum* under suitable conditions and also presents some characteristics of the process.

EXPERIMENTAL

Cultivation of the mold under conditions of agitation and aeration induces it to develop homogeneously throughout the medium in the form of suspended particles of mycelium and, depending on the nature of the medium, more or less in discrete small colonies. Under such conditions, the mold has optimum and homogeneous physiological conditions by the elimination of differential diffusion effects (see Kluyver and Perquin, 1933). These serve to accelerate markedly the rate of growth of *P. notatum* together with the formation of penicillin. The aeration may be secured by mechanical agitation (propeller or shaking machine) or by the passage of sterile air through the medium. In general, rather vigorous agitation is preferable.

Strain selection. Just as strain is the prime importance for maximum penicillin activity in surface cultures (Foster *et al.*, 1943), so it is in submerged penicillin production. Different strains vary markedly in penicillin-forming ability under submerged conditions, and strains best for surface production of penicillin are not necessarily best for submerged cultures, and vice versa. Table 1 summarizes several experiments, comparing different strains of *P. notatum* under submerged conditions, and emphasizes the importance of selecting a suitable strain for this type of process. A modified cup method for penicillin determination (Foster and Woodruff, 1943) was used throughout this work. Strain 832 has repeatedly proved to be far superior to the other strains tested. This culture was kindly furnished by Dr. R. D. Coghill of the Northern Regional Research Laboratories and was first found in that laboratory to be superior for submerged

¹ This manuscript was ready for publication in May, 1943, but was withheld from publication, material of this nature being under a U. S. Government secrecy order. See also footnote to paper VI of this series published in *J. Bact.*, 47, 43-58 (1944).

penicillin production. Strain 21, kindly supplied by Dr. George Harrop of the Squibb Institute for Medical Research and for a long time used in our laboratory for surface production of penicillin, is about one-half as effective as strain 832 in submerged cultures. The reverse, however, is true in surface cultures of these two strains (table 1).

TABLE 1
Penicillin formation by strains of Penicillium in surface and submerged cultures

	CULTURE	MAXIMUM PENICILLIN*		
		Submerged corn steep medium	Surface	
			Corn steep medium	Brown sugar medium
Expt. A	<i>P. notatum</i> 832 (NRRL)†	45	18	
	<i>P. notatum</i> 830 (NRRL)	20		18
	<i>P. notatum</i> 21 (144-5112.1)	8	38	22
Expt. B	<i>P. notatum</i> 832 (NRRL)	31	18	
	<i>P. notatum</i> 1892 (NRRL)	10	53	
	<i>P. notatum</i> 1249A (NRRL)	8	22	
	<i>P. notatum</i> 1249B21 (NRRL)	8	55	
Expt. C	<i>P. notatum</i> 21 (144-5112.1)	7		28
	<i>P. chrysogenum</i> (B.R.F.)‡	2		6
Expt. D	<i>P. chrysogenum</i> 140-5034.11§	18	40	
	<i>P. chrysogenum</i> 140-B472§		<4	
	<i>P. chrysogenum</i> 140-26§	8	32	
	<i>P. chrysogenum</i> 140-Ham 26§		28	
	<i>P. chrysogenum</i> 140-B508§		34	
	<i>P. chrysogenum</i> 140-94§		20	
	<i>P. chrysogenum</i> 140-B285A§	16	43	
	<i>P. chrysogenum</i> 140-5710.3§	13	40	
	<i>P. chrysogenum</i> 140-4733.33§		10	
	<i>P. notatum</i> 21		>40	
	<i>P. notatum</i> 832 (NRRL)	53		

* Oxford units per ml. Maximum value obtained from daily assays over a 7-day incubation period.

† Northern Regional Research Laboratory. We are grateful to Dr. R. D. Coghill for supplying the cultures marked NRRL.

‡ Biochemical Research Foundation. We wish to thank Dr. L. D. Smith for this culture.

§ We are indebted to Dr. Thom for these cultures.

Experiment B in table 1 compares under submerged and stationary conditions four cultures obtained from Dr. Coghill and again emphasizes the apparent inverse relation between the capacities to produce penicillin under these two methods of cultivation. Smith (1942) has shown that *Penicillium chrysogenum* produces penicillin. We have confirmed Dr. Smith's results with the strain of *P. chrysogenum* kindly furnished by him. That particular strain is a relatively poor producer of penicillin (experiment C). A number of other strains of *P.*

chrysogenum obtained through the courtesy of Dr. Charles Thom have also been tested (table 1, experiment D). Most of them produce penicillin in fairly large quantities in surface cultures. One strain, 140-B472, produced none under the test conditions. The four strains tested in submerged cultures produced penicillin at best in amounts decidedly inferior to the corresponding surface cultures. A strain of *Penicillium chlorophaeum* (kindly furnished by Dr. Thom) has also been found to produce penicillin. The conditions of cultivation have a marked influence on maximum penicillin formation in shake cultures since, for example, strain 21 has in other experiments formed considerably higher potencies than the 8 units per ml cited in table 1, experiment A.

Submerged culture experiments in synthetic media with P. notatum strain 21. Early experiments on penicillin production centered around synthetic media because the presence of complex organic supplements such as corn steep liquor, yeast extract, and *Penicillium* autolysate tended to yield penicillin preparations containing a considerably greater percentage of impurities than similar penicillin preparations obtained from synthetic media, this despite the higher initial

TABLE 2
Rise of pH and penicillin formation in submerged culture

INITIAL pH	PHOSPHATE BUFFER	pH			PENICILLIN, OXFORD UNITS/ML		
		6 days	10 days	12 days	6 days	10 days	12 days
6.7	1	6.8	8.1	8.2	3.5	>16.7	9
	5	6.7	7.0	7.7	4.5	11.3	5.5
	10	6.5	6.4	6.7	2.2	2.5	2
7.6	1	6.6	8.3	8.4	4.5	>16.7	9
	5	7.1		7.9	4.5		7
	10	7.3	6.8	7.7	2.8	3.5	2

potencies obtainable in complex media. The brown sugar medium of Hobby, Meyer, and Chaffee (1942) was found to be favorable, as it was in stationary cultures (Foster *et al.*, 1943). Ten grams per liter of NaNO_3 was considerably better than 3 grams, and in some experiments concentrations as high as 35 g per L seemed optimal. The reason for such high concentrations is not clear since they are far in excess of the nitrogen requirements of the mold. These experiments were conducted on machines shaking at the rate of 60 to 90 rpm, and maximum activity generally developed over a period of 6 to 10 days at 25 C. As in stationary cultures, a fall in pH occurs in the early days followed by a rapid rise up to 7.8 to 8.3. The formation of penicillin in those cultures bore a relation to the pH rise comparable to that for stationary cultures. Treatments in which the pH rose rapidly and attained a pH of 7.8 to 8.2 generally contained most penicillin. Table 2 shows the effect obtained as a result of buffering with different concentrations of potassium phosphate so that the pH rise could be controlled somewhat. Two sets at initial pH values of 6.7 and approximately 7.6 were run. Growth was abundant in all cases. The activities produced were roughly proportional to the degree to which the pH rose. It might be pointed out that the

relation existing between an ultimate high pH and penicillin formation is not necessarily absolute since, occasionally, activities of 40 to 60 Oxford units per ml have been obtained when the pH never exceeded 6.5 to 7.0.

The following synthetic medium was finally adopted for submerged penicillin production with *P. notatum*, strain 21: brown sugar, 60 g; NaNO_3 , 35 g; K_2HPO_4 , 16 g; KH_2PO_4 , 4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; distilled water, 1,000 ml. A number of other carbon sources can replace brown sugar without affecting the activities obtained. These include raw cane juice and high-test molasses. Blackstrap and invert molasses, cp sucrose and cp glucose, gave lower activities. NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , urea, *d*-glutamic acid, and NH_4 -acetate were satisfactory nitrogen sources, although NaNO_3 in general was best. Lower activities were obtained with asparagine, glycine, and peptone as the only sources of nitrogen.

Results were somewhat better when $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was added, although the effect was not very pronounced. In a brown sugar medium containing CaCO_3 , no increase in penicillin formation was obtained on adding soil extract or trace amounts of copper, manganese, or zinc, either singly or in combination. Zinc was required for maximum penicillin production in surface cultures of strain 21 (Foster *et al.*, 1943).

Chemical properties and antibacterial spectrum specificity tests proved that the penicillin extracted from such submerged culture filtrates is identical with that formed in surface cultures. Notatin, another antibacterial substance known to be formed by *P. notatum* in certain media in surface cultures (Coulthard *et al.*, 1942), has never been encountered in these experiments. Submerged cultures in corn steep liquor, brown sugar media invariably contain much less of the yellow pigment, chrysogenin, than do surface cultures. This is reflected in the product finally extracted.

Submerged culture experiments with P. notatum, strain 832, in synthetic media. In surface cultures the following modified brown sugar medium of Hobby, Meyer, and Chaffee (1942) is satisfactory for penicillin production with this strain (Foster *et al.*, 1943): dark brown sugar, 4 per cent; NaNO_3 , 0.6 per cent; KH_2PO_4 , 0.15 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 per cent; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ppm. This medium was distributed in 80-ml quantities in 250-ml Erlenmeyer flasks and inoculated with a spore suspension of *P. notatum* 832, and the flasks were incubated at 25 C on a shaking machine. Growth under such conditions commenced on the second day and, when maximum, appeared as small round pellets of mycelium, occupying about one-half the volume of the culture solution. Penicillin formation commenced on the third to fourth day and reached its maximum at 20 to 30 Oxford units per ml at 7 to 8 days. This contrasts with 9 to 14 days required for maximum activity in surface cultures.

As in surface cultures using synthetic media, an impure sugar was found essential for significant penicillin production. No activity was obtained with reagent sucrose or glucose, and the ash of brown sugar added to reagent sucrose medium could not replace brown sugar. Thus, brown sugar apparently contains an organic fraction responsible in part, at least, for the penicillin-promoting capacity of the former.

In surface cultures, as reported earlier from this laboratory, the addition of small amounts of zinc was necessary to obtain rapid oxidation and assimilation of sugar and to induce the pH to rise into the range favorable for penicillin production with strain 21 (Foster *et al.*, 1943). Zinc also has a similar catalyzing effect in submerged cultures of strain 832 (table 3). On the fifth day there were less than 3 units per ml in all treatments. Activity appeared in all treatments on the sixth day, and in the zero zinc set the activity was markedly less than in all the zinc-containing flasks. Best penicillin production was associated with the pH rise characteristic of the zinc cultures. The zero treatment had a pH considerably lower than the others. A minimum of 2 ppm $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 ppm Zn) was required for most rapid formation of penicillin. As noted for surface cultures, zinc-deficient cultures tend to approach the activity of zinc-containing cultures, but only after a considerably longer incubation period. The total cell material synthesized in the zinc-deficient cultures was appreciably less than that in the treatments. Only a slightly toxic effect was apparent with

TABLE 3
*Zinc and the formation of penicillin in submerged cultures**

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ mg per liter	6 DAYS		7 DAYS	
	Oxford units/ml	pH	Oxford units/ml	pH
0	3.6	5.9	11.6	6.8
1	14.4	7.4	21.4	7.4
2	20.4	7.7	28.0	7.6
5	19.6	7.7	21.0	7.7
10	20.8	7.4	22.4	7.7
25	18.6	7.0	16.8	7.6

* Brown sugar medium.

25 ppm zinc sulfate, whereas 10 ppm was definitely toxic in surface cultures. Other metals, including calcium, iron, manganese, copper, cadmium, iron, molybdenum, and tungsten, were without effect in cp sucrose medium and in brown sugar medium in concentrations of 0.1, 1.0, and 10 ppm.

Somewhat more than half the activity (17 Oxford units per ml) of the brown sugar medium was obtained when 4 per cent blackstrap molasses was used instead of brown sugar. Citrate, lactate, acetate, and glycerol could not replace brown sugar and led to no activity whatsoever, although growth was good. Among the various sources of nitrogen tested, nitrates were definitely superior. $(\text{NH}_4)_2\text{SO}_4$ and urea led to low activities. Various single amino acids and asparagine did not promote penicillin formation in the basal medium (with or without other N source).

Complex media—corn steep liquor. The beneficial effect of corn steep liquor in concentrations of 1 to 10 per cent on penicillin formation in surface cultures of *P. notatum* was first demonstrated in the Northern Regional Research Labora-

tory.² Some of the first experiments in our laboratory on submerged penicillin formation showed that corn steep liquor was also beneficial for activity under these conditions. However, the activities obtainable with the strain in use at that time were low enough to make the increased activity (4-fold) of questionable advantage because of the extra impurities invariably associated with the finally extracted penicillin product. This applied primarily in those cases in which the penicillin was desired particularly for chemical purification and structural studies. Later, however, as interest developed in a crude penicillin product for use in chemotherapy, effective despite its impure condition, the desirability of corn steep liquor for the process was indicated since more total unitage could be produced in this medium, though in a lower state of purity. In the meantime, the discovery of the greatly superior strain no. 832 by the Northern Regional Research Laboratory led to the resumption of penicillin production in corn steep media.

The optimum concentration of corn steep liquor varies from lot to lot because of the inconstancy of composition of this crude material. This refers to the total solids, titratable and reserve acidity, and quantitative composition. Generally speaking, the concentration is relatively unimportant providing it is about 1.0 to 1.5 per cent by volume. In several experiments no increase in penicillin production occurred with concentrations above 1.5 per cent. The introduction of corn steep liquor as a supplement to the regular 2 per cent brown sugar, 0.6 per cent NaNO_3 medium markedly accelerates the rate and total amount of penicillin produced. The entire volume of the medium becomes occupied with the filamentous mycelium of the mold, and the medium assumes a thick consistency in 2 to 3 days. Maximum penicillin activity is reached in 3 to 5 days, depending on the degree of aeration (see below).

All corn steep liquors are rather strongly acid. A good deal of the variation in effectiveness of different lots of corn steep liquor was traced to the differences in reserve acidity. It is necessary to determine the optimum neutralization conditions for any given lot of corn steep liquor (table 4). The particular lot of Staley brand of corn steep liquor (experiment A) had a relatively low reserve acidity. As a result of the growth of the mold, alkalinity develops, primarily because of the accumulation of the sodium ion left from the consumption of nitrate and the organic acids (lactic?) of the corn steep liquor. Neutralization to pH 6.0 to 8.0 led to the commencement of penicillin formation considerably earlier, and attainment of the maximum occurred one day earlier. Ultimately, the activities were all about the same high level after the initial low pH was raised by biological alkali formation. Experiment B, in which Clinton B corn steep liquor was used, led to only about one-half the activities of the Staley lot. The presence of CaCO_3 in the acid medium induced a beneficial effect beyond that obtainable by preliminary neutralization with soluble alkali.

The following list summarizes the conditions of neutralization required by various lots of corn steep liquor and emphasizes their variability.

² We are indebted to Dr. R. D. Coghill for the details on the stimulating effect of corn steep liquor.

<i>Type of corn steep liquor</i>	<i>Neutralization conditions for maximum penicillin formation</i>
Fermented* Corn Products.....	0.5-1.0 per cent CaCO_3
Fermented Clinton B.....	0.5-1.0 per cent CaCO_3 or NaOH to pH 8.0
Fermented Clinton A.....	0.5-1.0 per cent CaCO_3 indispensable
Nonfermented Clinton B.....	NaOH to pH 7.5 and 0.5-1.0 per cent CaCO_3
Nonfermented Clinton A.....	NaOH to pH 7.5 and 0.5-1.0 per cent CaCO_3
Nonfermented Staley.....	NaOH to pH 8.0 or 0.5-1.0 per cent CaCO_3

When these variations in reserve acidities are compensated for, little variation in penicillin rates and maximum could be obtained by concentrations of corn

TABLE 4
Neutralization of reserve acidity of corn steep media*
Experiment A. Staley brand. No CaCO_3

INITIAL pH	4 DAYS		5 DAYS		6 DAYS		7 DAYS	
	Oxford units/ml	pH	Oxford units/ml	pH	Oxford units/ml	pH	Oxford units/ml	pH
4.4	3	5.4	22	8.4	40	8.8	28	8.5
5.0	<3	4.9	23	7.7	38	8.4	32	8.7
6.0	9	5.7	39	8.2	39	8.5	29	8.8
7.0	8	6.0	38	8.0	39	8.5	31	8.7
7.5	10	6.1	41	8.1	40	8.5	40	8.7
8.0	15	6.2	40	8.3	45	8.6	38	8.6

Experiment B. Clinton B corn steep. No CaCO_3

INITIAL pH	OXFORD UNITS PER ML		
	3 days	4 days	5 days
4.5	<6	15	8
6.5	14	22	8
7.5	11	21	16

Clinton B corn steep. 1 per cent CaCO_3

INITIAL pH	OXFORD UNITS PER ML		
	3 days	4 days	5 days
4.5	<6	15	12
6.9	16	30	17
7.5	20	32	23

* Three per cent corn steep throughout.

steep liquor above 1.5 per cent. This summary, as well as table 3, indicates that nonfermented Clinton B corn steep liquor contains more substances which retard the rise in pH, which is generally associated with maximum penicillin formation.

* During some weeks' standing at room temperature, corn steep liquors undergo a slow spontaneous fermentation, which darkens them considerably.

Various carbohydrates are equally satisfactory for penicillin production in the presence of corn steep liquor, contrary to the results obtained in its absence. Table 5 shows typical results with different concentrations of brown sugar and glucose. Reagent sucrose and glycerol are as satisfactory as brown sugar. In general, little difference is obtained between 1 and 4 per cent carbohydrate. Concentrations higher than 4 per cent delay maximum penicillin formation and may reduce it somewhat. The amount of cell material synthesized under these conditions does not vary greatly with the sugar concentration. Thus, 1, 2, and 4 per cent carbohydrate cultures produced 0.81, 1.0, and 1.1 g dry cell material, respectively.

The relation between certain metabolic activities of *P. notatum* 832 in shake cultures is presented in figure 1. The medium consisted of brown sugar, 2 per cent; corn steep liquor (Staley), 3 per cent (vol.); CaCO_3 , 0.5 per cent; NaNO_3 , 0.6

TABLE 5
Effect of sugar concentration in corn steep medium

EXPT.	SUGAR	CONCENTRATION per cent	OXFORD UNITS PER ML		
			3 days	4 days	5 days
A	Brown sugar	0.5	17	20	10
	Brown sugar	1.5	46	37	20
	Brown sugar	2.0	67	37	16
B	Brown sugar	1.0	62	34	11
	Brown sugar	2.0	31	40	18
	Brown sugar	3.0	14	46	26
	Brown sugar	4.0	70	30	22
C	Glucose	1	41	35	18
	Glucose	2	27	38	17

per cent; KH_2PO_4 , 0.15 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 per cent; and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2 ppm. Samples from duplicate flasks were removed for analysis every 3 hours over a 78-hour period. The sucrose is rapidly inverted, so that after 12 to 24 hours all carbohydrate in solution is invert sugar. Mycelial weight and residual carbohydrate are roughly inversely proportional, and maximum growth and exhaustion of sugar occurred in this experiment in 42 hours. The initial lag is due primarily to spore germination. The rate of synthesis of mold cell material under these conditions is noteworthy. Extrapolating the log phase of growth, it is found that 1 g per 80 ml culture or 12.5 g per liter dry weight cell material was synthesized in about 30 hours. Based on the 1.60 g sugar originally present, this would represent a very high conversion, but the value is inaccurate because a considerable amount of the cell synthesis doubtless was made at the expense of the corn steep liquor, the consumption of which could not be measured. However, based on both the sugar and corn steep liquor consumed, the conversion

value probably is in the neighborhood of 40 per cent. Penicillin formation paralleled growth, but the maximum of the former occurred distinctly after the growth peak was attained. Penicillin formation increased almost 100 per cent after sugar consumption was complete. In many other experiments penicillin formation did not begin until sugar consumption was practically completed and rose rapidly to a peak 15 to 24 hours thereafter. The relation between growth and penicillin formation is not absolute since abundant growth may be obtained with little or no penicillin production. Activities as high as 80 units per ml are frequently obtained.

Only about a quarter of the nitrate originally present was assimilated. The sharp pH rise during the growth cycle is typical of submerged penicillin forma-

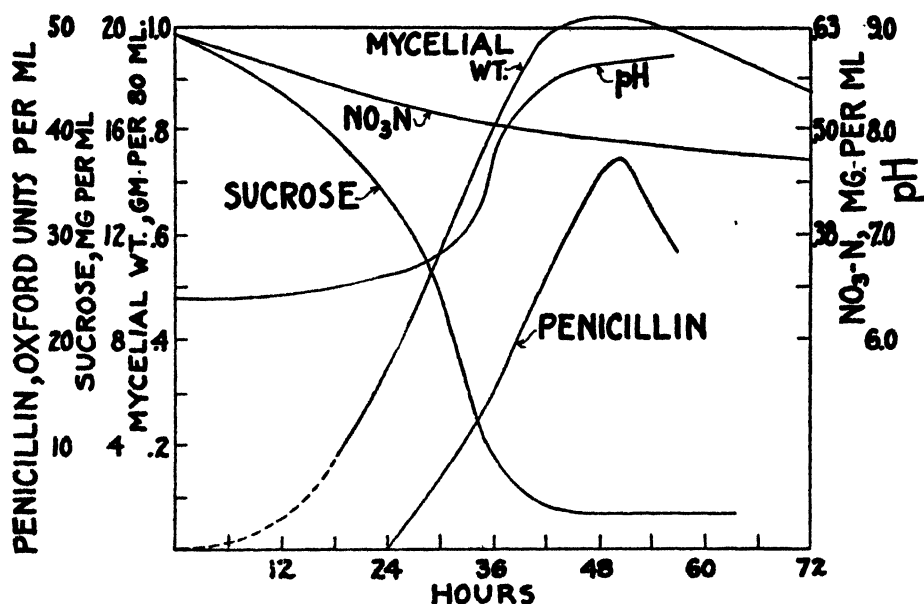


FIG. 1. METABOLIC ACTIVITIES IN SUBMERGED PENICILLIN CULTURE (CORN STEEP MEDIUM)

tion, as it is of surface cultures. This rise is due to the destruction by the mold of organic acid residues after the readily available and preferred energy source, namely, the sugar, is depleted. Residual inorganic cations such as Ca, K, and Na account for the alkalinity. The acidity arises from two sources: (1) organic acid produced by the oxidation of glucose, and (2) constituents of corn steep liquor, notably lactic acid and possibly other organic substances acting as buffers on the acid side. That the mold resorts to attacking the corn steep liquor as an energy source after the carbohydrate is exhausted is revealed by the liberation of ammonia at this stage, due to the utilization of proteins and protein split products. However, the relatively minute amounts of NH₃ formed obviously could not account per se for the sharp pH rise. At most only 0.05 mg ammonia per ml was found in this experiment.

Contrary to the foregoing results for synthetic media, NaNO_3 concentration is of little importance in corn steep media. Amounts from 0.1 to 0.6 per cent are equally good for maximum penicillin formation. In one experiment, 0.1 per cent NaNO_3 led to the production of 40 Oxford units per ml, whereas 20 were obtained in the absence of nitrate. Other salts commonly used in microbiological media, including KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, can be omitted in corn steep media without affecting penicillin production, since the corn steep liquor contains adequate amounts of these minerals. For example, in the absence of NaNO_3 , 18 units was the maximum production; with NaNO_3 , 37; and with NaNO_3 , KH_2PO_4 , and H_2SO_4 , 35. Zinc sulfate also is without effect in this medium, and produced toxic effects above 25 ppm. Ferrous sulfate up to 500 mg Fe per liter was inert with respect to penicillin formation.

Other complex supplements. Several other materials representing complex mixtures of organic substances, including ground whole seeds, sugar affinations, and concentrated distillery residue (whisky slop), hastened formation of peni-

TABLE 6
Organic supplements and penicillin production

SUPPLEMENT	OXFORD UNITS PER ML		
	3 days	4 days	5 days
3 per cent corn steep liquor.....	24	49	31
3 per cent affinations.....	13	<8	10
3 per cent soybean meal.....	14	12	12
3 per cent cottonseed meal.....	19	38	24
3 per cent corn meal.....	16	16	20
3 per cent ground rice.....	6	10	16
10 per cent of 1-4 whisky slop extract.....	13	16	17

cillin over that usually obtained in the basal brown sugar medium, although in most cases maximum activity was no greater than that obtained in the basal medium (table 6). Cottonseed meal approached corn steep liquor in promoting penicillin formation and eliminated the need for neutralization. It was selected for further study.

Different lots of cottonseed meal show considerable variation in results. Certain lots, selected after preliminary testing, definitely promote penicillin formation in amounts as low as 0.1 per cent (table 7).

Routine examination of cultural conditions with cottonseed meal media, by variations in the qualitative and quantitative composition of the medium, revealed a particularly interesting finding which has a bearing on the growth and penicillin-promoting properties of brown sugar and of cottonseed meal. Table 8, typical of repeated experiments, shows that cp sucrose, cottonseed medium is unfavorable for penicillin formation, being no better than cp sucrose synthetic medium, i.e., without the organic supplement. However, when the ash, left upon ignition of an equivalent amount of brown sugar, was added to cp sucrose, cottonseed medium, it could replace the favorable effect of brown sugar in cotton-

seed medium. Brown sugar ash had no effect in cp sucrose in the absence of cottonseed. It may be that the ash of brown sugar supplies certain minerals

TABLE 7
Cottonseed meal and penicillin formation

	COTTONSEED MEAL	OXFORD UNITS PER ML		
		4 days	5 days	6 days
Type A	<i>per cent</i>			
	0	<6	<8	
	0.1	10	34	
	0.5	28	21	23
	1.0	36	30	23
Type B	2.0	30	30	19
	0.5	28	40	28
	1.0	38	25	36
	2.0	23	24	13

TABLE 8
Effect of brown sugar ash in cottonseed meal medium

SOURCE OF CARBOHYDRATE (2 PER CENT IN EACH CASE)	OXFORD UNITS/ML		
	3 days	4 days	5 days
Brown sugar.....	14	30	18
Sucrose (cp).....	<6	<8	<8
Sucrose (cp) and ash of brown sugar.....	8	27	21

TABLE 9
Penicillin-promoting factors in brown sugar and cottonseed meal

MEDIUM	FACTORS SUPPLIED AND SOURCE	MAXIMUM ACTIVITY OXFORD UNITS PER ML
Sucrose (cp).....	None	0
Brown sugar.....	Ash and organic	20
Sucrose (cp) + cottonseed....	Organic	0
Sucrose.....		30-40
brown sugar ash.....	Ash	
cottonseed.....	Organic	
Brown sugar.....	Ash and organic	30-40
cottonseed.....	Organic	
Brown sugar.....	Ash and organic	20
cottonseed ash.....	Ash	
Sucrose.....		40-60
corn steep.....	Ash and organic	
Brown sugar.....	Ash and organic	40-60
corn steep.....	Ash and organic	

in the optimum balance required for penicillin formation. This indicates that at least two types of factors are responsible for promoting penicillin formation,

one organic and one inorganic. The ash of cottonseed meal does not replace the organic portion of the meal. Both factors (or ones acting similarly) are present in brown sugar, since the promoting effect is obtained in brown sugar synthetic medium, but not in cp sucrose synthetic medium, nor in the latter containing the ash of brown sugar. Destruction of the organic fraction in brown sugar by ignition renders cp sucrose medium unfavorable for penicillin formation, but does not affect cottonseed medium since the organic fraction is supplied in the cottonseed. The latter, however, does not supply the required ash constituents. These relationships are summarized in table 9. As the total amount of mold mycelium synthesized in cottonseed media is invariably substantially less than that in corn steep media, more penicillin is synthesized per unit of cell material in cottonseed medium than in corn steep medium.

Penicillin production in pilot plant fermenter. The submerged process using culture 832 in corn steep liquor was next tried out on a pilot plant scale with very successful results. A 75-gallon capacity fermenter of a vertical stationary type, constructed of carbon steel and having propeller agitation, was charged with 200 liters of medium of the following composition:

Corn steep liquor.....	3	per cent by volume
Brown sugar (soft, dark) ..	2	per cent
NaNO ₂	0.6	per cent
KH ₂ PO ₄	0.15	per cent
MgSO ₄ ·7H ₂ O.....	0.05	per cent
ZnSO ₄ ·7H ₂ O.....	1	ppm
CaCO ₃	0.25	per cent (added as sterile slurry after sterilization of the batch)

The batch was sterilized 1 hour at 120 C. Agitation was 230 rpm, air flow was 150 cu ft hr, and the fermentation was run under 5 pounds of air pressure. Sterile tributyl citrate was added aseptically whenever needed as an antifoam, in quantities to suppress foam. The inoculum consisted of a spore suspension from 6 Roux cultures of *P. notatum*, strain 832. The following tabulation summarizes the data on this run:

DATE	AGE	OXFORD UNITS PER ML	pH
	<i>hours</i>		
9/14/42	0		
9/17/42	72	21	6.5
9/18/42	88	29	7.6
9/18/42	96	36	
9/20/42	120	50	

Later, by means of a vegetative inoculum, maximum activity was obtainable in less than 3 days.

Aeration and penicillin formation in submerged cultures. The shake or submerged culture technique described above is designed to facilitate better aeration

than surface culture allows. Fungi are essentially aerobic organisms, and oxidation of sugars for energy and cell synthesis, prerequisites for penicillin formation, is greatly accelerated by optimum supplies of air. Pure oxygen prevents growth of *P. notatum* from a spore inoculum.

In the basal brown sugar medium, aeration is not apt to be a limiting factor, because of the slow and limited amount of growth generally obtained in this medium. With organic supplements, such as corn steep or cottonseed, excessively rapid and abundant mycelial development creates a very high requirement for oxygen. Table 10 contains data indicating that aeration may be a limiting factor in penicillin formation. Not only are higher activities obtained with the best aeration achieved by more rapid agitation, but the whole process of growth and penicillin formation is speeded up to the extent that daily assays are no longer sufficient to establish curves of growth and penicillin formation. Invariably, penicillin formation is so rapid, and the activities drop off so rapidly after the peak is reached, that erroneous conclusions may be obtained concerning the

TABLE 10
Aeration and penicillin formation

RELATIVE AGITATION*	3 DAYS		4 DAYS		5 DAYS		6 DAYS	
	Oxford units per ml	pH	O.u./ml	pH	O.u./ml	pH	O.u./ml	pH
Fast.....	22	7.5	35	8.1	<8		<8	8.4
Medium.....	12	7.1	24	7.9	13	8.4	<8	8.5
Slow.....	<8	5.7	16	7.4	21	8.0	11	8.2

* Rotary shaking. The rate of agitation is an index of the degree of aeration. These values are approximate and were obtained arbitrarily by multiplying rpm by eccentricity measured in inches.

efficacy of treatments, since sampling once daily may or may not coincide with the time of peak penicillin accumulation. A rotary shaking speed of 228 rpm (faster than that reported in table 10) reduces the time required for peak penicillin formation to 60 hours and doubles the maximum activity, yielding up to 80 Oxford units per ml in this time. In certain experiments the penicillin content of the cultures increased at the rate of 5 Oxford units per ml per hour.

SUMMARY

Growth of suitable *Penicillium notatum* strains in shake culture (submerged growth developed by agitation and aeration) leads to the rapid formation of potent penicillin broths. This method of culturing has numerous advantages over surface cultures: the variable factors of diffusion and pellicle formation are eliminated, and growth and metabolic processes are accelerated.

A determining factor in submerged culture work is the selection of a suitable strain of penicillin-producing mold.

Penicillin formation takes place in shake culture in a nearly synthetic medium

in which brown sugar supplies certain substances essential for growth and penicillin formation.

Crude organic supplements hasten the development of *P. notatum* and increase the maximum penicillin titer obtained. Corn steep liquor serves as an excellent supplement, provided the reserve acidity of this material is adjusted with NaOH, CaCO₃, or both. Sugar concentration has slight effect upon penicillin formation. Maximum penicillin formation is obtained after the disappearance of the sugar from the medium.

Other organic supplements, particularly cottonseed meal, promote penicillin formation in the same manner as corn steep.

Evidence is presented that there are two factors promoting penicillin formation, one inorganic ash and one organic in nature. Brown sugar contains both, and cottonseed meal contains at least the organic factor.

Paramount in importance in the production of penicillin in submerged culture is the maintenance of an adequate oxygen supply.

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BIOLOGIC CHANGES IN SULFONAMIDE-RESISTANT MYCOBACTERIUM RANAE

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The present report describes the development of a sulfonamide-resistant strain of *Mycobacterium ranae* which grows at a slower rate than the "parent" susceptible strain and differs in certain other important respects.

I. PRODUCTION OF SULFONAMIDE-RESISTANT STRAIN OF MYCOBACTERIUM RANAE

The microorganism employed in these studies was *Mycobacterium ranae*, a rapidly growing acid-fast bacillus which is nonpathogenic for mammals, grows over a wide range of temperature variation, and reaches a maximum growth on Long's liquid, synthetic medium in 5 days at pH 7.2 and 37 C. It grows on the surface of Long's medium, producing a creamy white, moderately wrinkled pellicle, and no discoloration of the subnatant medium. The organisms were also grown on glycerol nutrient agar for some experiments on solid medium. The sensitivity of this strain to sulfonamide is constant as was proved by the failure of many transfers over a period of 1 year to grow in the presence of 1 mg per cent or more of sulfathiazole (ST).

The development of a strain highly resistant to ST was accomplished by serial transfer through Long's medium containing increasing concentrations of the drug. Each transfer was performed by picking from the fully grown surface pellicle one standard 5-mm loopful of pellicle. The drugs were added to the media before autoclaving at 15 pounds for 15 minutes.

EXPERIMENTAL

A sulfonamide-resistant strain of *M. ranae* which gave luxuriant growth in the presence of 200 mg per cent ST was produced after 23 transfers during a period of 3 months. This culture differed from the parent susceptible strain in the following respects:

(1) The resistant strain continued to maintain its high resistance unchanged after 35 transfers in plain Long's medium over a period of 10 months.

(2) There was a parallel increase in resistance to all other sulfonamide and sulfonamidelike agents tested—sulfanilamide, sulfapyridine, sulfadiazine, and diaminodiphenylsulfone.

(3) During the course of the development of the resistant strain, it was observed that the culture medium became more and more golden in color and the pellicle more granular in appearance. The golden color of the subnatant medium changed to a light orange after the resistant strain had been transferred in plain Long's medium for six transfers, but it was not possible by further transfers to

cause a return to the clear, colorless medium of the parent susceptible strain, although the granularity of the surface did disappear.

The light orange medium in which the resistant strain had grown was tested by the conventional method for sulfonamide antagonism and was found to possess no more sulfonamide antagonism than the colorless medium in which the parent susceptible strain had grown. The colored medium was tested in the usual manner for diazotizable arylamine by Bratton and Marshall's method and was found to contain much more diazotizable amine than the medium in which the parent susceptible strain had been grown. The organisms themselves were tested for the same thing and were found also to contain much more diazotizable arylamine than the parent susceptible strain (Yegian and Budd, 1945).

(4) It grew at a rate approximately one-half as fast as the parent strain, even after 30 transfers in Long's medium without sulfathiazole. The reduction in rate of growth of the resistant strain was verified by the following observation: Long's synthetic medium and glycerol nutrient agar were inoculated from a suspension (turbidity no. 1 MacFarland's nephelometer) containing sensitive and resistant organisms in equal proportion. The maximum growth from this culture was resuspended in buffer solution, diluted to a no. 1 nephelometer reading, and transferred to fresh medium. After five such transfers the resistant strain could not be recovered. In subsequent tests it was found that even in a mixed population in which the proportion of resistant to susceptible cells was as great as 99 to 1, the growth rate of the sensitive form was evidently so rapid that the resistant organisms could eventually be entirely weeded out.

The following procedure was adopted to determine whether or not resistance to sulfonamide could be developed in the absence of cell multiplication: Cell suspensions (corresponding to no. 1 MacFarland nephelometer) from a 2-day-old culture of *M. ranae* grown on Long's medium were placed in phosphate buffer solutions (pH 7.2) containing graded concentrations of ST varying from 0.5 to 25 mg per cent. These suspensions were kept at 37 C, and at 10-day intervals, over a period of 3 months, transfers of one standard loopful were made on glycerol agar containing corresponding concentrations of the drug. The organisms constantly failed to grow in the presence of as little as 1 mg per cent ST, indicating thereby that cell multiplication is essential to the acquirement of sulfonamide resistance. Viability was not greatly impaired as was shown by the abundant growth obtained when transfers were made to glycerol agar containing 1 mg per cent of *para*-aminobenzoic acid.

The observations of Griffith (1928), Dawson (1930), and Avery *et al.* (1944) on the transformation of pneumococcus types led us to attempt to transform the resistant strain back to the susceptible form and vice versa. One strain was cultivated in media containing the heat-killed bacillary bodies of the other strain for seven successive transfers. The experiment was also attempted *in vivo* by intraperitoneal inoculation of mice, but no reversals of type were observed on testing the final sulfonamide sensitivities.

In order to test whether or not there were any differences in antigenic structure (immunological specificity) between the parent susceptible and the resistant

strains, rabbits were immunized with each strain and their sera tested for agglutinins. Identical cross agglutinations in high titers were observed. Agglutinin-absorption tests also failed to reveal any immunological differences between the two variants.

There were no differences in the morphology of the parent susceptible and the resistant strains as revealed by the Ziehl-Neelsen technique.

DISCUSSION

The observation that microorganisms can be induced to develop a permanent chemoresistance to sulfonamides is not new, but the development of a resistant variant the growth rate of which is markedly and consistently slower than that of the parent susceptible strain is of some interest. This allows the conclusion, based upon the described observations, that there is no tendency of the resistant strain to mutate back to the original susceptible strain, for if it did this, even infrequently, the latter would soon outgrow the former. The reverse cannot be stated, however, and it still remains to be shown whether the acquisition of drug fastness is one of selection of normally occurring heredity variants, present as such in the original cell population, or of the specific induction of such forms by the sulfonamide. The fact that organisms exposed to sulfonamide under conditions precluding multiplication failed to develop resistance to the drug indicates that cell division is essential for the development of resistance.

The slower growth rate, different color of the medium, and production of increased arylamine by the resistant strain point to some metabolic change or changes which should be amenable to biochemical investigation and may reveal some facts of significance in the mode of action of the sulfonamides. Such changes apparently have not been reported to occur with other microorganisms which have been rendered resistant to the sulfonamides.

The failure of the discolored medium of the resistant strain to antagonize sulfonamide bacteriostasis shows the difference between this organism and organisms studied by other investigators (Green, 1940; Green and Bielschowsky, 1942; MacCleod, 1940), although a resistant strain of *Staphylococcus aureus* has been observed (Spink and Vivino, 1943) to show a similar change, which, however, was not maintained in the absence of the sulfonamide.

The failure to cause a genetic reversal of sulfonamide sensitivity by the technique effective with pneumococcal type-specific polysaccharides does not necessarily mean that eventual understanding of the heredity-determinant mechanisms will not lead to the reversal of resistant to susceptible strains of microorganisms.

SUMMARY

Mycobacterium ranae has been rendered permanently sulfonamide-resistant by the conventional method *in vitro*. The resistant strain differs from the parent susceptible strain in many important respects including growth rate, discoloration of the medium, and production of diazotizable arylamine.

It was deduced that the sulfonamide-resistant strain was a true and stable mutant.

The discolored medium had no special sulfonamide-antagonistic activity although it gave the Bratton and Marshall test for arylamine.

A resistant mutant could not be induced in an environment in which there was no cell multiplication.

A reversion of strains could not be induced by the technique effective with pneumococci with regard to type specificity.

No change in immunological specificity was demonstrable by the usual technique.

II. SULFONAMIDE RESISTANCE AND SUSCEPTIBILITY TO ANTIBACTERIAL AGENTS

It seemed of interest to determine whether or not there were any differences in the susceptibility of the parent susceptible strain and the resistant strain of *Mycobacterium ranae* to agents other than the sulfonamides. The experiments described below were designed for this purpose.

The cylinder-plate method used in penicillin assay (Abraham, Chain, *et al.*, 1941) was found satisfactory for demonstrating the comparative effects of various agents on sulfonamide-resistant and sulfonamide-susceptible *M. ranae*.

One standard drop of a bacillary suspension (turbidity no. 1 MacFarland's nephelometer) of 3-day-old cultures of the test organisms was distributed evenly over the surface of glycerol nutrient agar plates. The agent to be tested was placed in a glass cylinder, which was tightly imbedded on the surface of the agar, and the plates were incubated at 37 C for 4 to 5 days. The diameter of the zone of inhibition was then measured with a millimeter rule.

EXPERIMENTAL

It was necessary to employ a concentration of the chemical agent which permitted some peripheral growth of both the susceptible and the resistant strains. Duplicate tests were always performed, and, although the diameters of the inhibited areas were not identical, the difference between the susceptible and the resistant organisms was clearly demonstrable in every instance. These differences were not considered significant unless obviously greater than the error inherent in the tests. Table 1 shows the average results of the cylinder-plate tests.

The plate method, being dependent upon the diffusibility and the bacteriostatic power of the agent, is not a measure of germicidal efficiency (Tobie and Ayres, 1944). However, for the purpose of comparing the resistance of the two strains of *M. ranae* to the various agents, the method is simple and satisfactory.

The slower rate of growth of the sulfonamide-resistant organism suggested the possibility that the delay might permit wider diffusion of the chemical agent and thus account for the observed differences in some instances. Therefore, plate tests were set up which permitted diffusion of the chemicals before multiplication began. With this technique, the differences between the sensitive and the resistant organisms were identical with the results presented in the table.

A further check on the possibility that the rate of diffusion of the chemicals from the cup into the agar determined the observed differences in susceptibility

was made by pour plates of glycerol agar with various concentrations of the agents. It was found that the highest concentration of oxalic acid permitting some growth of the susceptible strain was 1:1,500, whereas the sulfonamide-resistant strain failed to grow at a concentration of 1:2,000. In a medium containing copper sulfate the sensitive parent strain grew well at 1:2,000 as compared with poor growth of the resistant organisms at 1:2,800.

DISCUSSION

It is evident from an examination of table 1 that the sulfonamide-susceptible strain was in general more resistant to the antibacterial effect of the group of agents tested than was the sulfonamide-resistant strain. This appears to be entirely nonspecific, being true for bases, acids, and to a greater or lesser extent for the wide variety of agents tested. The nonspecific nature of the difference

TABLE 1
Resistances of strains of Mycobacterium ranae

AGENT TESTED	PERCENTAGE OF CONCENTRATION	DIAMETER OF ZONE OF INHIBITION IN MM	
		Sulfonamide-resistant <i>M. ranae</i>	Sulfonamide-susceptible <i>M. ranae</i>
Oxalic acid.....	4.5	59	42
Nitric acid.....	6.3	62.5	54
Potassium hydroxide....	5.0	18	14
Copper sulfate.....	16.0	69	54
Phenol.....	2.0	26	15
Barium chloride.....	20.0	23	15
Zephiran.....	0.1	47	41
Pyridium.....	1.0	40	35
Azochloramid.....	0.033	20	12
Gentian violet.....	1.0	43	33
Formalin.....	10.5	25	20
Tr. metaphen.....	0.12	68	53
Acriflavin.....	0.1	50	44

suggests that it is related to the difference in growth rates of the two strains. The slower multiplication of the sulfonamide-resistant strain would allow each cell a longer period of exposure to the antibacterial agent (or environment) before its division. This may explain the observed differences.

It has been observed (Emmart and Smith, 1942) that virulent tubercle bacilli rendered resistant to the bacteriostatic effect of promin, a sulfonamidelike agent, produced fewer lesions on the chorio-allantoic membrane of the chick embryo than the original parent promin-susceptible strain of tubercle bacilli. This was interpreted as indicating a reduction in virulence of tubercle bacilli when they become sulfonamide-resistant. It is suggested on the basis of these observations with *M. ranae* that the apparent reduction in virulence was due to a slowing of the growth rate of the tubercle bacilli. Unfortunately no observations with regard to the comparative growth rate of the promin-susceptible and promin-resistant strains *in vitro* were reported.

SUMMARY

A sulfonamide-resistant strain of *M. ranae* was found to be nonspecifically more susceptible to antibacterial agents (antagonistic environment) than its parent sulfonamide-susceptible strain. It is suggested that this is in some way associated with its slower growth rate. The rates of multiplication of microorganisms should be considered in the comparison of the susceptibilities of various strains to antibacterial agents or other unfavorable environmental factors.

III. SULFONAMIDE RESISTANCE AND PARA-AMINOBENZOIC ACID ANTAGONISM

The well-known antagonism of *para*-aminobenzoic acid in small amounts to sulfonamide bacteriostasis has been the subject for investigations into the mechanism of bacteriostasis by sulfonamides. Various theories have been advanced to explain this antagonism and the biochemical mechanisms involved. In an attempt to throw additional light on this subject, sulfonamide-resistant and parent sulfonamide-susceptible strains of *M. ranae* were utilized.

Parent susceptible and resistant strains were tested for their susceptibility to bacteriostasis by PABA. The growth rate of the parent susceptible strain in Long's medium was reduced to approximately one-half that of the controls by a concentration of 400 mg per cent PABA; on the other hand, the sulfonamide-resistant strain was almost completely inhibited by the same concentration of PABA.

This difference in the behavior of the susceptible and resistant strains remained constant after repeated transfers of the organisms over a period of 7 months.

DISCUSSION

The foregoing observation indicates that the mechanisms of bacteriostasis by sulfonamides and PABA are different, since the sulfonamide-resistant strain is less resistant to bacteriostasis by PABA than the parent susceptible strain. The theory, therefore, that PABA antagonism of sulfonamide inhibition of bacterial multiplication is of the nature of an "ionic exchange" in which two substances, separately toxic for some specific phase of bacterial metabolism, completely or in part nullify each other's effects when mixed in proper balance (Henry, 1943) is open to question. According to this theory, a strain of bacteria rendered resistant to sulfonamide bacteriostasis should also have an increased resistance to PABA bacteriostasis. Such is not the case with the strain studied here. Indeed, the sulfonamide-resistant strain is somewhat more susceptible to bacteriostasis by PABA than is the parent sulfonamide-susceptible strain. This seems to place PABA in the large group of nonspecific antibacterial agents, previously described, all of which were more active against the sulfonamide-resistant strain of *M. ranae*.

In view of the fact that repeated exposure of a culture of microorganisms to any growth-inhibitory agent usually induces the development of a resistant variant, it is of some significance that it was not possible to render *M. ranae* resistant to PABA by multiple transfers. This observation also gives further evidence that bacteriostasis by sulfonamides and by PABA depends upon different mechanisms.

It is of theoretical and, perhaps, of practical import that a sulfonamide-resistant strain of microorganisms could not be reverted to a sulfonamide-susceptible strain by prolonged cultivation in the presence of PABA. If the development of sulfonamide resistance simply depends on the ability of the microorganisms to produce more PABA which can compete with the sulfonamide, as has been suggested (Landy *et al.*, 1943), it would seem possible that that strain could be induced by high concentration of PABA to produce less PABA and thus become more susceptible to sulfonamide inhibition. That this could not be done indicates the complexity of the mechanisms of induced variations of bacterial metabolism.

SUMMARY

A sulfonamide-resistant variant of *Mycobacterium ranae* has been shown to be slightly more susceptible to bacterostasis by *para*-aminobenzoic acid than the parent sulfonamide-susceptible strain.

It was not possible to produce a change in the susceptibility of *M. ranae* to bacteriostasis by PABA or to sulfonamides by repeated transfers in a medium containing large amounts of PABA.

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ULTRASONIC DISINTEGRATION AS A METHOD OF EXTRACTING BACTERIAL ENZYMES¹

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In recent years several techniques have been developed for the purpose of disintegrating bacterial cells. These have depended upon different principles ranging from autolytic rupture of the cell to grinding with powdered glass (Lipmann, 1938; Koepsell and Johnson, 1942; Wiggert *et al.*, 1940; Kalnitsky *et al.*, 1945; Booth and Green, 1938). Although each method has merit for some specific problem, it frequently proves inadequate for other applications.

Of the several methods employed in this laboratory, that of disintegrating bacterial cells by exposure to an ultrasonic field² has proved of most value when limited quantities of bacteria were available and when the possibility of preparing cell-free enzyme extracts had to be explored. Further, since irradiation of bacteria by ultrasound can be carried out under completely aseptic conditions, it becomes possible to analyze pathogenic bacteria with respect to their endotoxins, enzymes, polysaccharides, and other substances which can be extracted from cells only after disintegration. The method, however, has two drawbacks: (1) the construction of the apparatus is expensive, and (2) not all bacteria can be disintegrated by ultrasound.

APPARATUS

As a detailed account of the ultrasonic equipment employed in this laboratory has been published elsewhere (Smith and Stumpf, 1946), only a brief description is given here. An X-cut quartz crystal³ (1½ inches in diameter and ground to a frequency of 660 kilocycles) is driven by a radio-frequency generator, operating at 2,000 volts with a rated output of 700 watts. The generator has a frequency range from 200 to 1,000 kilocycles and employs beam power tetrodes to eliminate neutralization. It has the usual crystal controlled exciter as well as a variable frequency exciter, which is stabilized and calibrated, and which facilitates exploring a wide range of radio frequencies. Figure 1 is a diagrammatic representation of the complete unit and the disintegrating chamber, respectively.

An efficient crystal mounting must fulfill several requirements, among which are low mechanical damping of the crystal by the holder, high insulation against

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² The application of sonic energy to biological problems is not new (cf. Chambers and Flosdorf, 1936), but previously no systematic effort has been made to explore the technique from the standpoint of extracting bacterial enzymes.

³ We are indebted to the August E. Miller Laboratories and the Federal Telephone and Radio Corporation for quartz crystals.

voltage breakdown, and ready accessibility to permit rapid change of operating frequency. With these requirements in mind, we developed a crystal holder such

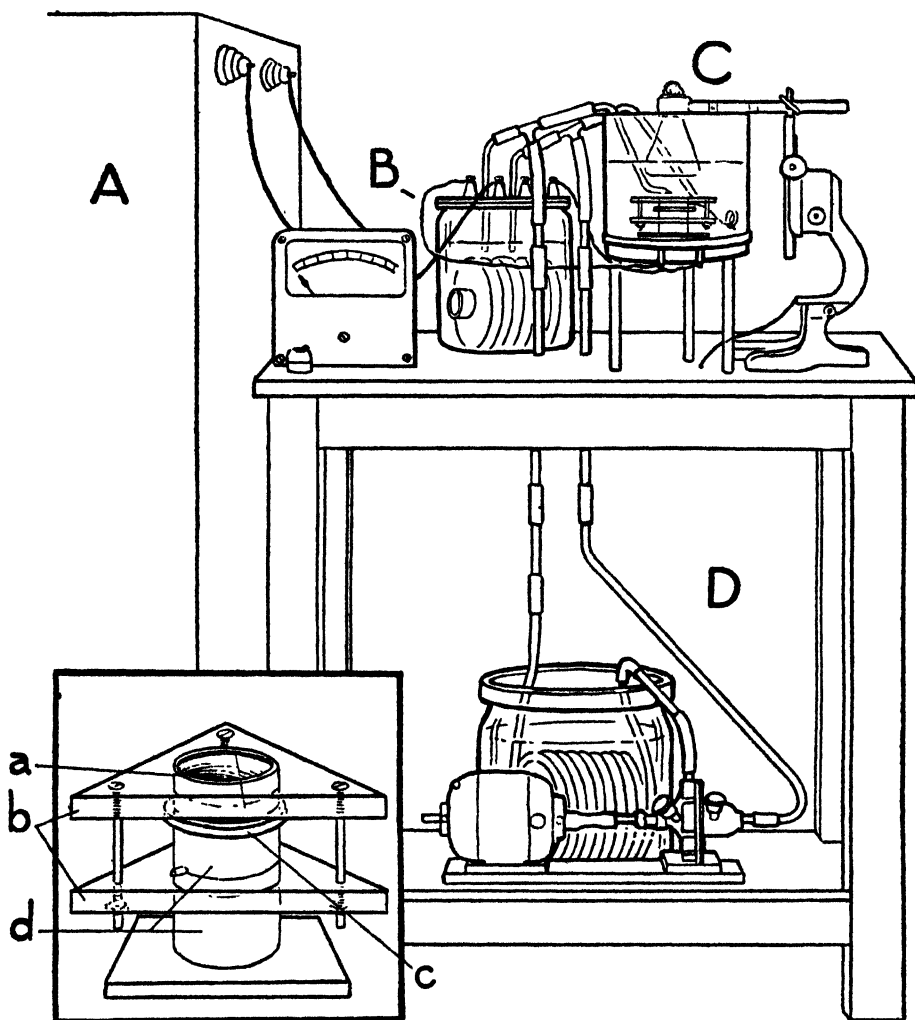


FIG. 1

- A. Radio-frequency transmitter.
- B. Coupling coil.
- C. Disintegration chamber and rack and pinion arrangement.
- D. Cooling arrangement for oil in circulating system.
- a. Upper brass electrode.
- b. Adjustable lucite plates for holding electrodes in place.
- c. Quartz crystal.
- d. Lower brass electrode.

as is shown in figure 1. It consists of a quartz crystal sandwiched between two brass rings held together by a lucite press device which permits changes in con-

tact pressure of the brass electrodes on the quartz surfaces. Although it leads to some damping, this arrangement has the advantage that breakdown of the insulating medium is eliminated completely. With other types of holders which were constructed and tested, voltage breakdown of the transformer oil resulted in immediate and irreparable damage to the surface of the quartz crystal. This surface is silver-plated routinely every 6 months by Brashear's method (Hodgman, 1937). It was found that a silver-plated surface is superior to one of aluminum or silver foil cemented or spattered onto the quartz. To increase the voltage breakdown path, the upper and lower plating is not carried out to the extreme edge of the crystal. Lucite is used exclusively in the mounting and in the oil chamber since, as insulation against high voltage, it is superior to either neoprene or bakelite.

The crystal holder is completely immersed in transformer oil,⁴ which serves at once as insulation medium, conducting medium for ultrasound, and circulating fluid for the cooling system. The oil is circulated continuously by a centrifugal pump from the ultrasound apparatus through a copper coil surrounded by an ice-water mixture. Much heat is formed during operation, and efficient cooling is necessary to prevent the temperature from rising above 30 C in the bacterial suspension.

When the crystal holder is immersed in the oil bath, the lower brass ring electrode automatically forms an air pocket, which almost completely reflects the sound waves at the lower side of the quartz plate. The reflected waves come automatically into the right phase with those radiated upwards and consequently increase their energy (Bergmann and Hatfield, 1938). Hence the total damping of the crystal becomes less, owing to the decrease in the radiation decrement.

The bacteria to be disintegrated were made up in a thin suspension containing about 20 mg dry weight per ml and were placed in a 50-ml Erlenmeyer flask, which was then lowered by a rack and pinion device into the oil chamber to a critical distance from the upper face of the quartz crystal. The optimum distance is reached when the cone of fluid in the flask is at maximum height during ultrasonic irradiation. A 10-minute exposure usually ensures satisfactory disintegration. The suspension is spun for 10 minutes at 20,000 rpm in the high-speed conical head of the refrigerated International centrifuge no. 1. After centrifugation, three layers are observed: a supernatant fluid which is faintly opalescent and pale yellow, and a sediment consisting of two layers, (a) a lower, hard-packed layer of intact cells and (b) an upper, gelatinous layer of ghost cells and debris. The supernatant fluid is removed with a pipette, care being taken not to disturb the sediment. The same technique is employed when pathogens are disintegrated. It is of course necessary to follow aseptic procedures in handling pathogenic material.

The supernatant fluid still contains a residual number of intact, viable cells, but the number is so small that these do not contribute significant enzymic activity to the juice. This conclusion is most easily demonstrated by considering the enzymic activity of the supernatant fluid of a suspension of disintegrated cells

⁴ Wemco "C" transformer oil obtained from Westinghouse Electric Supply Co., New York.

of *Proteus vulgaris* with respect to pyruvic oxidase (Stumpf, 1945). Whereas the whole cell rapidly oxidizes pyruvic acid without addition of any factors, the supernatant juice from disintegrated cells is completely inert unless cocarboxylase is added (as indicated in figure 2). From this fact it follows that the number of intact cells is too low to account for any appreciable fraction of the observed enzyme activity. As a precaution against gross bacterial contamination, however, the extracted juice is centrifuged daily in the high-speed attachment before its use and is always kept chilled at about 4 C. In some cases it may be lyophilized and stored at 0 C.

THE FACTORS WHICH AFFECT THE EFFICIENCY OF ULTRASONIC DISINTEGRATION

A number of factors have been considered which determine the maximum utilization of ultrasonic energy for the disintegration of bacteria. Two factors

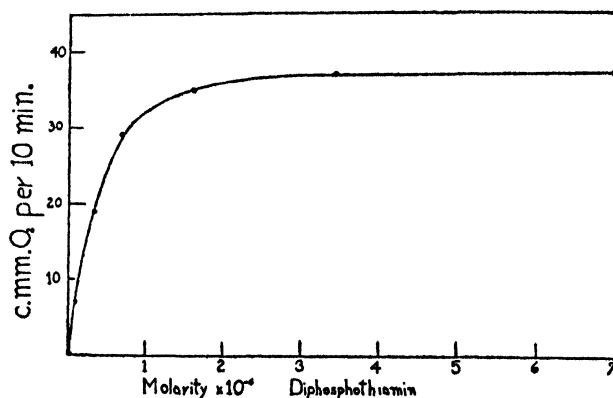


FIG. 2. REACTION VELOCITY OF CELL-FREE ENZYME PREPARATION OF PYRUVIC OXIDASE OF *PROTEUS VULGARIS* AS A FUNCTION OF DISPHOSPHOTHIAMINE CONCENTRATION

Each manometric cup contained 0.5 ml of enzyme, 0.5 ml of $M/2$ acetate buffer at pH 6.0, 0.1 ml of 0.2 per cent manganese sulfate, 0.5 ml of $M/5$ lithium pyruvate. Final volume 3 ml; NaOH in center pot, temp 38 C.

which come immediately to mind—namely, time of exposure and shape of container—can be treated briefly. As indicated in table 1, after 10 minutes' ultrasonic irradiation, the maximum degree of disintegration is reached. The suspension probably contains forms of the bacterial cell which are resistant to the disruptive forces, and hence complete disintegration is not possible. As for the second factor, it has been our experience that vessels such as test tubes or round-bottom flasks, because of their convex bases, reflect and thereby dissipate a large percentage of the ultrasonic radiation. An ideal container should therefore have a perfectly flat, thin glass base which would permit a maximum passage of disruptive energy. Routinely, we employ small Erlenmeyer flasks or volumetric flasks which have relatively thin bases.

As shown in table 2, the thickness of the suspension, or viscosity, is a factor of considerable importance. A thick paste of bacteria suffers little or no disinte-

gration of cells. When the suspension is diluted, however, disintegration by ultrasound takes place. Evidently, when a suspension exceeds a certain viscosity, ultrasonic radiation which strikes such a suspension is largely converted to heat, which has no disruptive effect.

TABLE 1
Relation between time of exposure and degree of disintegration of E. coli

EXPOSURE TIME	MG OF NITROGEN PER ML OF ORIGINAL SUSPENSION	MG OF NITROGEN PER ML OF CELL-FREE "JUICE"	DEGREE OF DISINTEGRATION*
<i>minutes</i>			<i>per cent</i>
2.5	0.94	0.19	25
5	0.94	0.30	40
10	0.94	0.43	56
15	0.94	0.47	63

* $\frac{\text{mg of nitrogen per ml cell-free juice}}{\text{mg of nitrogen per ml original suspension}} \times \frac{100}{0.80}$. The theoretical limit for this ratio is approximately 80 per cent, since ghost cells and cellular debris which contribute the remaining 20 per cent of nitrogen are removed by centrifugation.

TABLE 2
Relation between concentration of bacteria and degree of disintegration of E. coli

Bacteria		
A. MG BACTERIAL N PER ML OF SUSPENSION	B. MG BACTERIAL N PER ML OF CELL-FREE "JUICE"	DEGREE OF DISINTEGRATION 100B/0.80A
		<i>per cent</i>
2.18	0.44	25
1.37	0.37	34
0.63	0.20	39
0.32	0.13	50

Erythrocytes

DILUTION OF SUSPENSION	TIME FOR COMPLETE HEMOLYSIS
	<i>seconds</i>
Thick paste of undiluted centrifuged cells.....	No hemolysis
1:5 (dilution with isotonic salt solution).....	75
1:10 (dilution with isotonic salt solution).....	50
1:15 (dilution with isotonic salt solution).....	25
1:20 (dilution with isotonic salt solution).....	20
1:25 (dilution with isotonic salt solution).....	7

When the distance between the upper face of the crystal and the base of the container is such that the suspension is mildly agitated by ultrasonic energy, the degree of disintegration is about half that which is obtained when the distance is critical and the suspension is violently agitated (table 3). Obviously, when the distance between the upper face of the quartz plate and the base of the container

is a multiple of the whole wave length of the ultrasonic vibration, any reflection from the base of the container will cause partial interference. When, however, the distance traveled by the waves is an odd multiple of one-half the wave length, reinforcement of the reflected wave occurs and considerably more ultrasonic energy is carried into the suspension. As shown in table 4, a considerable number of cells readily yield to treatment, but others are completely refractory to ultrasound.

Finally, the relative degree of disintegration of bacteria seems to be independent of the frequency of the ultrasonic wave and dependent only on the intensity or amplitude of the wave. It is unfortunate, however, that, if the power output

TABLE 3

The position of the irradiating flask with reference to the quartz crystal as a factor in determining the degree of disintegration

DISTANCE OF FLASK FROM QUARTZ CRYSTAL	STATE OF BACTERIAL SUSPENSION DURING IRRADIATION	MG OF BACTERIAL NITROGEN PER ML OF SUSPENSION	MG OF BACTERIAL NITROGEN PER ML OF CELL-FREE "JUICE"	DEGREE OF DIS-INTEGRATION
				<i>per cent</i>
1 mm shift from the critical position.....	Gentle agitation	0.92	0.14	19
Critical position.....	Vigorous agitation	0.91	0.29	40

TABLE 4

Organisms exposed to ultrasonic irradiation

EASILY DISINTEGRATED		REFRACTORY
<i>Hemophilus influenzae</i>	<i>Proteus vulgaris</i>	<i>Sarcina lutea</i>
<i>Salmonella typhi-murium</i>	<i>Clostridium welchii</i>	<i>Micrococcus lysodeikticus</i>
<i>Brucella abortus</i>	<i>Escherichia coli</i>	<i>Acetobacter suboxydans</i>
<i>Lactobacillus casei</i>	<i>Pseudomonas pyocyanea</i>	<i>Saccharomyces cerevisiae</i>
<i>Lactobacillus delbrueckii</i>	<i>Pseudomonas fluorescens</i>	(Chloroplasts)
<i>Proteus morganii</i>	<i>Staphylococcus aureus</i>	
	(Erythrocytes)	

is raised beyond a certain range, denaturation and inactivation of labile proteins rapidly takes place. Thus there is actually only a relatively narrow region of power intensity in which ultrasonic energy can be effectively used as a disintegrating agent.

SUMMARY

A method of disintegrating bacterial cells by ultrasonic radiation is described. The method has been employed to prepare cell-free enzyme extracts of bacteria. A number of factors which influence the degree of efficiency of ultrasonic disintegration are discussed.

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EXTERNAL OTITIS, WITH ADDITIONAL STUDIES ON THE GENUS *PSEUDOMONAS*¹

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Bacterial or fungous infections of the ear canal have been recognized and described repeatedly. However, there has been a pronounced difference of opinion as to the principal etiologic organisms and the exact sources of infection. Since the authors were in contact with many cases of external otitis, a study of 100 consecutive new or acute cases was undertaken in order (a) to determine the relative frequency of fungi and bacteria as infective agents, (b) to learn of any possible relationship between the severity of the condition and the type of organism isolated, and (c) to provide material for further investigations on the characteristics of the principal pathogenic organism or organisms. No cases of chronic otitis externa were included in this series.

I

Some investigators (Minchew, Collins, and Harris, 1940) claim that there is no appreciable difference in the bacterial flora of pathologic ear canals as compared with that of the normal, and therefore that fungi (chiefly members of the genus *Aspergillus*) are the important etiologic organisms. Others, such as Gill (1938, 1942), Whalen (1938), and Dobes (1943), have also emphasized the importance of fungi in this disease. On the other hand, the results of Williams, Montgomery, and Powell (1939) indicate that bacteria are the most prominent organisms in diseased ear canals, with fungi present in only a small minority of cases. A few authors have indicated the possible role of *Pseudomonas* types as the causative agent in otitis externa (Morley, 1938; Dagget, 1942; Davis, 1943).

Materials and Methods

After the ear canal had been sponged with 70 per cent alcohol and dried with sterile cotton, samples for culturing were obtained by scraping the epithelium with a small, sterile curette and by streaking the resultant material on three different media: Saboraud's maltose agar (for the isolation of fungi), 0.5 per cent glucose blood agar (for the cultivation of hemolytic bacteria), and Difco nutrient agar (for the isolation of less fastidious organisms). The tubes and plates were then incubated at room temperature for 24 to 96 hours before examination. The resulting growth, when fungal, was examined after fixation and staining in a

¹ The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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lactophenol cotton blue solution; when bacterial, after staining with methylene blue and Gram's stain. Some of the isolated bacteria, notably members of the genus *Pseudomonas*, were further studied.

Data were obtained from each of the patients concerning (1) his original home locality, (2) age, (3) sex, (4) race, (5) history of previous ear pathology, (6) present residence, (7) recent or pertinent water contacts, including swimming pools, and (8) symptoms of disease at the time of first reporting. In each case, in order to standardize therapeutic methods, the treatment was the same—namely, gentle cleansing of the ear canal followed by application of tincture of merthiolate to the affected area and dusting with a powder consisting of two parts of sulfathiazole, two parts of sulfanilamide, and one part of sodium perborate. No additional treatment was used. In those instances in which pain was intense, hot compresses were applied for 30 minutes four times a day until pain was relieved.

Results

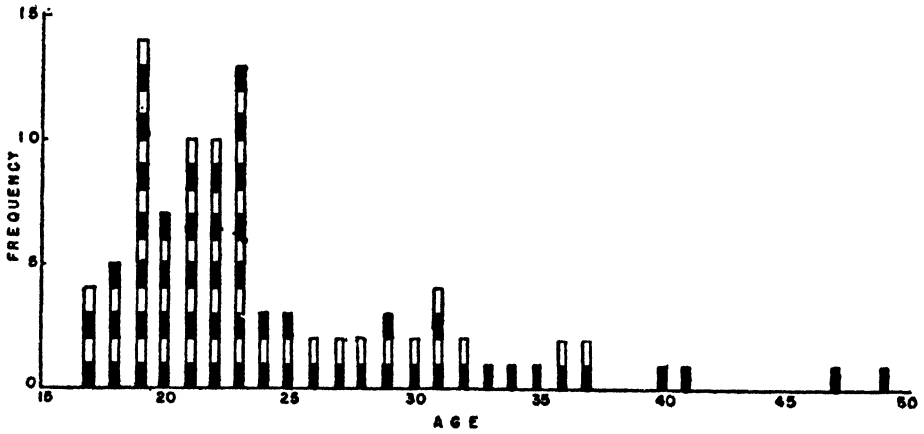
Of the 100 cases observed, 44 were limited to the right ear, 30 to the left, and 26 involved both ears. Of those that had otitis externa in the left ear alone, 9 were left-handed, whereas the rest, who had right ears or both ears pathologic, were right-handed. Five per cent of the patients were colored, the rest white, although 18 per cent of the personnel aboard were colored. The ages, as shown in graph 1, varied from 17 to 48 inclusive, with the great majority occurring in the 17- to 23-year-old group.

Of interest, especially in view of the simple, relatively mild medication used, are the data of the severity of the infection and the time for cure. Symptoms were classified according to the amount of swelling, discharge, and pain, with additional notes concerning the presence of itching and the color of the discharge. Each patient was examined every 24 to 48 hours, and the initial dates of onset and disappearance of pathologic symptoms were noted.

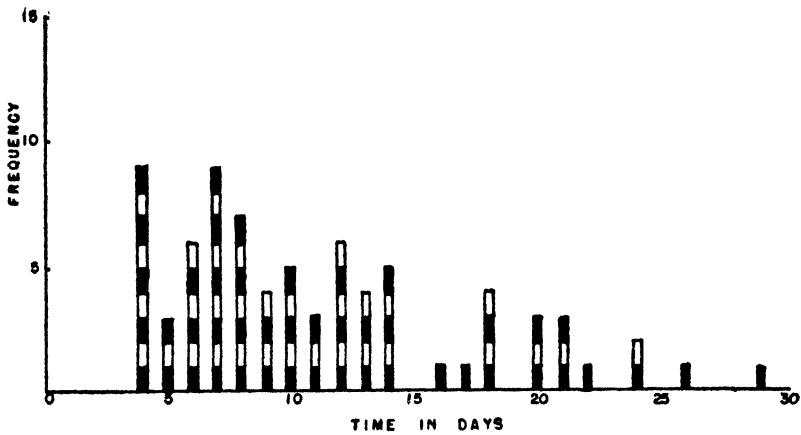
The results (graph 2) show that the time for complete cure, symptomatically and bacteriologically, varied from a minimum of 4 days to a maximum of 29, with the mean at 11.2 days. Of those ears from which fungi were isolated the time varied from 4 to 14 days, with the mean at 4.5 (graph 3). The ears from which species of *Pseudomonas* were isolated required from 4 to 29 days, with the mean at 12.7 days (graph 4). Those individuals who had a previous history of otitis externa were cured in a mean time of 8.9 days, whereas those who had not were cured in a mean time of 12.3 days (graphs 5 and 6). Bacterial infections also produced more severe symptoms of pain, swelling, and discharge than those of fungi, there being but slight difference in the effect of different bacteria (graph 7).

Attempts were made to identify all the organisms obtained from the infected canals. Of the 100 cases, bacteria alone were isolated from 84, fungi alone from 8, and a mixture of fungi and bacteria from 8. Of the 16 fungal isolates, 1 was a species of *Monotospora*; 4 were *Actinomyces israeli*; 3, *Aspergillus niger*; 4, *Aspergillus flavus*; and 4, *Aspergillus terreus* (table 1).

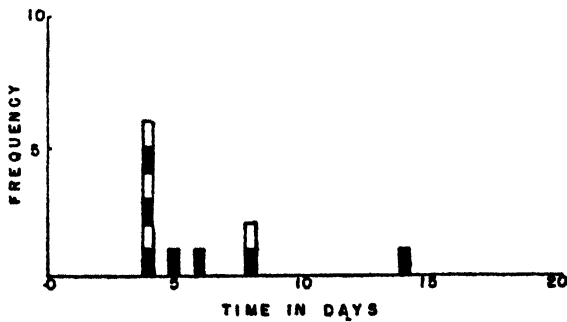
Of the patients from whose ears bacteria were isolated, 45 had *Pseudomonas*



GRAPH 1. ILLUSTRATION OF THE NUMBER OF INDIVIDUALS OF AGES FROM 17 TO 49 WITH OTTIS EXTERNA

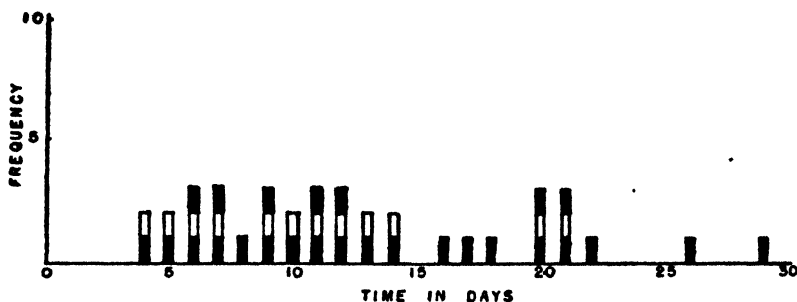


GRAPH 2. ILLUSTRATION OF THE TIME REQUIRED FOR CURE FROM EXTERNAL OTTIS

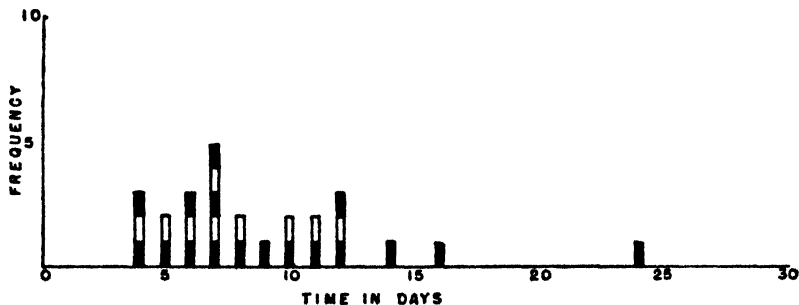


GRAPH 3. ILLUSTRATION OF THE TIME REQUIRED FOR CURE IN THOSE FROM WHOSE EARS FUNGI WERE ISOLATED

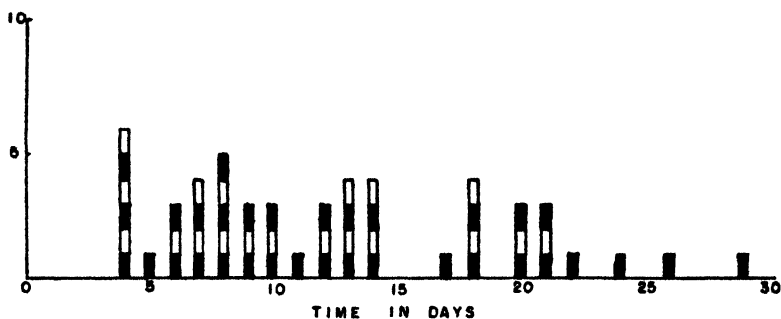
sp.; 27, *Staphylococcus albus*; 14, unidentified diphtheroids; 9, viridans streptococci; 9, *Chromobacter* sp.; 2, *Sarcina lutea*; 2, hemolytic streptococci; and 1, *Staphylococcus aureus* (table 2).



GRAPH 4. ILLUSTRATION OF THE TIME REQUIRED FOR CURE IN THOSE FROM WHOSE EARS *Pseudomonas* Sp. WERE ISOLATED



GRAPH 5. ILLUSTRATION OF THE TIME FOR CURE AMONG THOSE WHO HAD PREVIOUSLY HAD EXTERNAL OTITIS



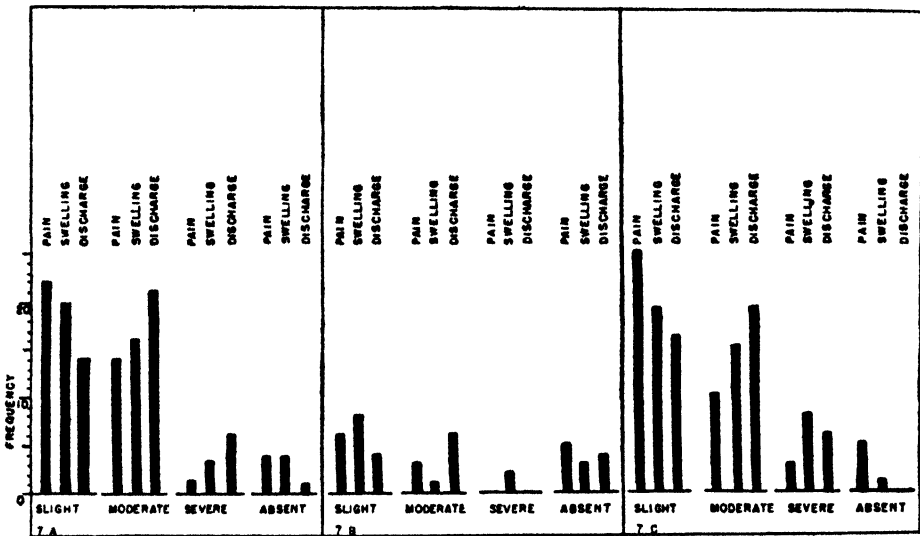
GRAPH 6. ILLUSTRATION OF THE TIME FOR CURE AMONG THOSE WHO HAD NOT PREVIOUSLY HAD EXTERNAL OTITIS

Cultures were taken from 25 normal ear canals in the same way as from pathologic ones, and yielded the following results: gram-negative rods, 17; *Staphylococcus albus*, 16; and no organism, 4. Fungi were not isolated from normal ear canals.

Analysis of Data

When the foregoing data are analyzed, several deductions can be made. The time for the cure of a fungus infection of the ear canal is noticeably less than that of a bacterial infection, notwithstanding the current opinion of the marked severity of mycotic invasions of the ear. The ease with which fungus in the ear canal was eradicated was in marked contrast, for example, to the definite persistence of some of the *Pseudomonas* sp. infections, this occurring during the midsummer months when the mean temperature was 82.4 F and the average relative humidity 78.7 per cent. These fungous infections usually had a fairly copious, colored discharge, and exhibited relatively little pain and swelling.

As shown in graphs 5 and 6, those individuals who had a history of otitis externa were cured on the average in less time (8.9 days) than those who had no



GRAPH 7. ILLUSTRATION OF THE SYMPTOMS OF EXTERNAL OTITIS CAUSED BY (A) *Pseudomonas* SP., (B) FUNGI, AND (C) OTHER ORGANISMS ISOLATED

history (12.3 days). This may be significant, although it is pointed out here merely to suggest possibilities for future control of the ailment.

No causal relationship was found between individual age and the occurrence of external otitis, since, as was to be expected from naval personnel, most of the patients were in the 17- to 26-year age bracket. Also, no unusual results were seen in the number or type of recurrence in the "cured" cases, wherein only 9 per cent of the patients returned with very mild infections during a period of 3 months following the termination of the original infection.

The type of organism that was isolated from the diseased ear was noticeably different from that found in the normal ear. Of course, according to the experiments completed, there is no certainty that the organisms isolated were the causative ones, but the very fact that the flora differs is an indication that the

organisms concerned are either primarily or secondarily related to the onset of the disease.

Of outstanding interest are the small percentage of fungi obtained and the relative ease with which they responded to treatment. *Pseudomonas* sp., which occurred in 45 per cent of the diseased canals and in none of the normals, was the most frequently isolated organism and the one associated with the cases least responsive to treatment. *Staphylococcus albus*, which was the most common organism in normal ears, was isolated from 27 per cent of the pathologic ears, although frequently it occurred with other forms. A yellow *Chromobacter* sp.

TABLE 1
Fungi isolated from infected ear canals
(100 patients)

IDENTITY OF ORGANISM	NUMBER OF PATIENTS
<i>Actinomyces israeli</i>	4
<i>Aspergillus terreus</i>	4
<i>Aspergillus flavus</i>	4
<i>Aspergillus niger</i>	3
<i>Monotropa</i> sp.....	1

TABLE 2
Bacteria isolated from infected ear canals
(100 patients)

IDENTITY OF ORGANISM	NO. OF PATIENTS FROM WHOM ISOLATED		
	In pure culture	In mixed culture	Total isolations
<i>Pseudomonas</i> sp.....	32	13	45
<i>Staphylococcus albus</i>	15	12	27
Diphtheroids.....	14	0	14
Viridans streptococci.....	6	3	9
<i>Chromobacter</i> sp.	0	9	9
<i>Sarcina lutea</i>	1	1	2
Hemolytic streptococci.....	0	2	2
<i>Staphylococcus aureus</i>	0	1	1

was also found in 9 per cent of the diseased canals, generally in conjunction with other bacteria.

II

Since *Pseudomonas* sp. was most frequently isolated from diseased ear canals, further studies were made of this organism. *Pseudomonas aeruginosa* has been recognized as a potential human pathogen for many years, and yet little is known about its properties. Its most striking characteristic is its formation of pyocyanin, a chloroform-soluble, nonfluorescent, blue pigment. However, there is a

striking difference of opinion as to its fermentative action and its other biochemical characteristics.

For example, on page 20 of the pamphlet of the Enteric Pathogen Laboratory, National Naval Medical Center (1944), there is the statement, "*Pseudomonas aeruginosa* is easily recognized culturally due to the fact that with the exception of a slight and transient acidity in glucose, all carbohydrate media are rendered alkaline." Moltke (1927) and Bergey *et al.* (1939) drew similar conclusions, but others such as Topley and Wilson (1936) and Stitt *et al.* (1938) believe that glucose alone is attacked. Clara (1934), on the other extreme, claimed that his isolate of *P. aeruginosa* fermented glucose, galactose, levulose, salicin, mannose, arabinose, xylose, mannitol, and glycerol. Elrod and Braun (1942) follow an intermediate path in asserting that only glucose, xylose, and arabinose were fermented.

Biochemical studies were therefore made (1) to study the extent of the fermentative activity of 56 isolates of *Pseudomonas* sp.; (2) to determine the exact type or types of *Pseudomonas* sp. isolated from the ear according to Bergey's classification of this genus; and (3) to compare strains obtained from four different sources, namely, diseased ear canals, pathologic stools, drinking water, and soil.

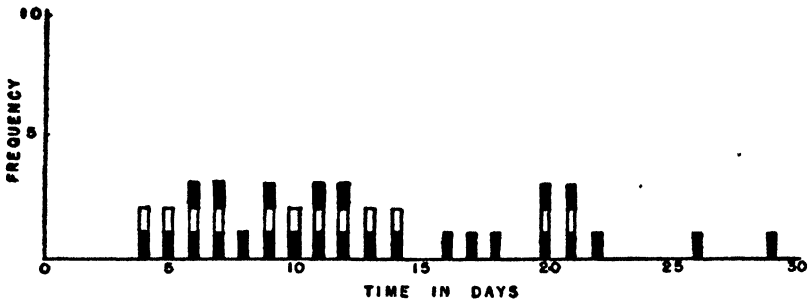
The cultures tentatively identified as *Pseudomonas aeruginosa* because of the production of the pigment pyocyanin were isolated from the following sources: 13, from diarrheal stools, in which no other established enteric pathogen could be found; 28, from pathologic ear canals; and 2, from chlorinated well water. Thirteen other isolates were made from soil, and they all were included as *P. fluorescens* because of the presence of fluorescein and the absence of pyocyanin.

Each isolate was first examined for motility after 24-hour growth in Difco nutrient broth at 37 C, and all were established as definitely possessing that character. These observations on the motility of the organisms were further substantiated by 24-hour growth at 37 C on a semisolid medium made by the mixture of two solutions: one consisting of 80 g bacto gelatin in 600 ml of water; and the other of 5 g sodium chloride, 10 g bacto peptone, 3 g Liebig's beef extract, 4 g bacto agar in 400 ml of water. Again, all the organisms showed definite motility, with isolates S-1 and S-2 forming additionally a contrastingly large amount of blue-green pigment.

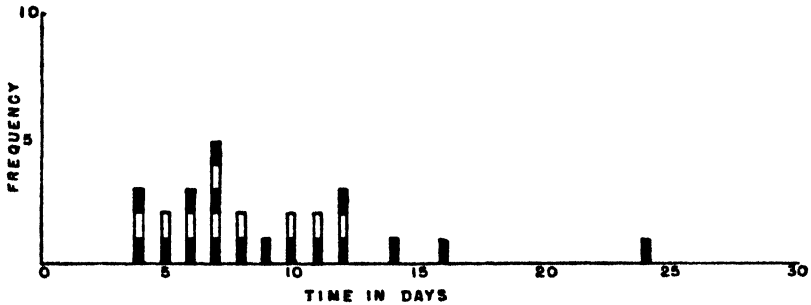
After 21 days' incubation at room temperature in 1 per cent peptone water, none of the 56 isolates formed hydrogen sulfide or indole. After 5 days' growth at 37 C in Difco nutrient broth containing 0.1 per cent potassium nitrate, 6 of the 13 *P. fluorescens* group and 21 of the 43 *P. aeruginosa* cultures reduced nitrate to nitrite when tested by the Griess-Ilosva method. None of the cultures showed any evidence of the formation of nitrogen gas in Durham tubes.

Ten per cent gelatin stabs of the isolates were made and kept at room temperature for 28 days. All the *P. aeruginosa* group that reduced nitrate liquefied gelatin with infundibuliform type of growth in 3 to 15 days, whereas those that did not reduce nitrate either displayed a stratiform-filiform type of liquefaction (8 in number) or none at all (2 in number). Three of the *P. fluorescens* group showed a stratiform-filiform type of liquefaction, and the rest none at all, with

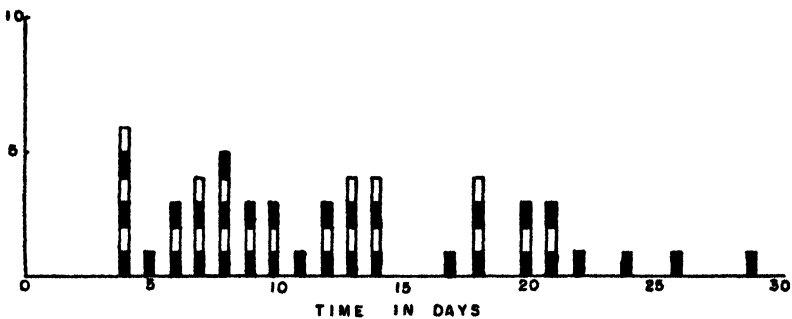
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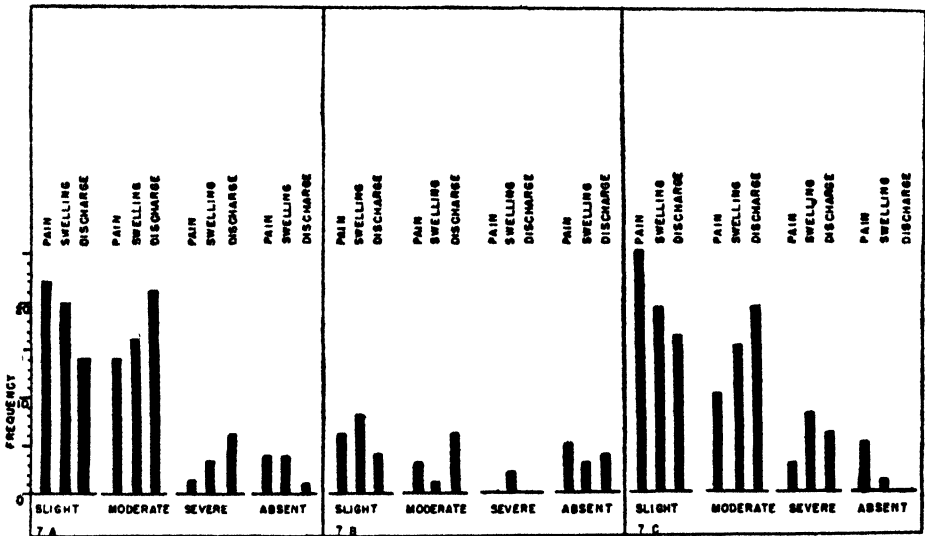
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(100 patients)

IDENTITY OF ORGANISM	NUMBER OF PATIENTS
<i>Actinomyces israeli</i>	4
<i>Aspergillus terreus</i>	4
<i>Aspergillus flavus</i>	4
<i>Aspergillus niger</i>	3
<i>Monotospora</i> sp.....	1

TABLE 2
Bacteria isolated from infected ear canals
(100 patients)

IDENTITY OF ORGANISM	NO. OF PATIENTS FROM WHOM ISOLATED		
	In pure culture	In mixed culture	Total isolations
<i>Pseudomonas</i> sp.....	32	13	45
<i>Staphylococcus albus</i>	15	12	27
Diphtheroids.....	14	0	14
Viridans streptococci.....	6	3	9
<i>Chromobacter</i> sp.....	0	9	9
<i>Sarcina lutea</i>	1	1	2
Hemolytic streptococci.....	0	2	2
<i>Staphylococcus aureus</i>	0	1	1

was also found in 9 per cent of the diseased canals, generally in conjunction with other bacteria.

II

Since *Pseudomonas* sp. was most frequently isolated from diseased ear canals, further studies were made of this organism. *Pseudomonas aeruginosa* has been recognized as a potential human pathogen for many years, and yet little is known about its properties. Its most striking characteristic is its formation of pyocyanin, a chloroform-soluble, nonfluorescent, blue pigment. However, there is a

striking difference of opinion as to its fermentative action and its other biochemical characteristics.

For example, on page 20 of the pamphlet of the Enteric Pathogen Laboratory, National Naval Medical Center (1944), there is the statement, "*Pseudomonas aeruginosa* is easily recognized culturally due to the fact that with the exception of a slight and transient acidity in glucose, all carbohydrate media are rendered alkaline." Moltke (1927) and Bergey *et al.* (1939) drew similar conclusions, but others such as Topley and Wilson (1936) and Stitt *et al.* (1938) believe that glucose alone is attacked. Clara (1934), on the other extreme, claimed that his isolate of *P. aeruginosa* fermented glucose, galactose, levulose, salicin, mannose, arabinose, xylose, mannitol, and glycerol. Elrod and Braun (1942) follow an intermediate path in asserting that only glucose, xylose, and arabinose were fermented.

Biochemical studies were therefore made (1) to study the extent of the fermentative activity of 56 isolates of *Pseudomonas* sp.; (2) to determine the exact type or types of *Pseudomonas* sp. isolated from the ear according to Bergey's classification of this genus; and (3) to compare strains obtained from four different sources, namely, diseased ear canals, pathologic stools, drinking water, and soil.

The cultures tentatively identified as *Pseudomonas aeruginosa* because of the production of the pigment pyocyanin were isolated from the following sources: 13, from diarrheal stools, in which no other established enteric pathogen could be found; 28, from pathologic ear canals; and 2, from chlorinated well water. Thirteen other isolates were made from soil, and they all were included as *P. fluorescens* because of the presence of fluorescein and the absence of pyocyanin.

Each isolate was first examined for motility after 24-hour growth in Difco nutrient broth at 37 C, and all were established as definitely possessing that character. These observations on the motility of the organisms were further substantiated by 24-hour growth at 37 C on a semisolid medium made by the mixture of two solutions: one consisting of 80 g bacto gelatin in 600 ml of water; and the other of 5 g sodium chloride, 10 g bacto peptone, 3 g Liebig's beef extract, 4 g bacto agar in 400 ml of water. Again, all the organisms showed definite motility, with isolates S-1 and S-2 forming additionally a contrastingly large amount of blue-green pigment.

After 21 days' incubation at room temperature in 1 per cent peptone water, none of the 56 isolates formed hydrogen sulfide or indole. After 5 days' growth at 37 C in Difco nutrient broth containing 0.1 per cent potassium nitrate, 6 of the 13 *P. fluorescens* group and 21 of the 43 *P. aeruginosa* cultures reduced nitrate to nitrite when tested by the Griess-Ilosva method. None of the cultures showed any evidence of the formation of nitrogen gas in Durham tubes.

Ten per cent gelatin slabs of the isolates were made and kept at room temperature for 28 days. All the *P. aeruginosa* group that reduced nitrate liquefied gelatin with infundibuliform type of growth in 3 to 15 days, whereas those that did not reduce nitrate either displayed a stratiform-filiform type of liquefaction (8 in number) or none at all (2 in number). Three of the *P. fluorescens* group showed a stratiform-filiform type of liquefaction, and the rest none at all, with

no apparent correlation between nitrate reduction and the type of liquefaction.

Each of the isolates was grown in Difco nutrient broth at both 42 C and 5 C, according to the suggestions of Seleen and Stark (1943). All of the isolates showed heavy growth at 42 C with the exception of the soil series, which showed no growth after 5 days' incubation. At 5 C, on the contrary, the soil group exhibited light to heavy growth after 31 days, whereas the rest of the isolates showed either very scanty growth or none at all.

When the different organisms were inoculated onto potato plugs, which had previously been soaked in 1 per cent sodium carbonate for 30 minutes, growth was luxuriant in all cases after 7 days at room temperature, with most of the pyocyanin-producing forms exhibiting a brown, yellow, and blue-green pigmentation, and the others a pale to deep yellow one. Similarly, when the isolates were grown in litmus milk at room temperature for 7 days, peptonization, clotting, and an alkaline medium were produced by most of the pyocyanin-forming groups, and merely an alkaline medium by all but one of the soil isolates.

Fermentation studies were made on the 56 strains of *Pseudomonas* sp. wherein the following carbohydrates were tested: adonitol, arabinose, dextrin, glucose, dulcitol, fructose, galactose, glycerol, inositol, inulin, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sorbitol, starch, sucrose, trehalose, and xylose. Since *Pseudomonas* sp., when growing in proteinaceous media, produces ammonia, which in turn would mask the formation of acid, a medium was used in which ammonia production was at a minimum. This synthetic medium, suggested by Elrod and Braun (1942), consisted of 0.2 g magnesium sulfate, 0.1 g calcium chloride, 0.2 g sodium chloride, and 0.2 g dipotassium phosphate per liter of distilled water. As an example, 43 of the isolates were grown both in Difco phenol red broth (proteose-peptone no. 3, 10 g; bacto beef extract, 1 g; sodium chloride, 5 g; and bacto phenol red, 0.018 g per liter) and in the Elrod-Braun synthetic medium with either one of several sugars added, and the fermentative activity was compared. The results clearly demonstrate that, since in the phenol red broth only 18.6 per cent of the isolates in glucose and 81.4 per cent in xylose acidified the solution, as contrasted with 95.3 per cent in the synthetic medium, the fermentative reactions of a species of *Pseudomonas* should not be studied in the presence of organic nitrogen.

The 43 isolates which produced pyocyanin all fermented glycerol; with the exception of 2 strains, all fermented arabinose, glucose, galactose, mannose, and xylose; but none acted upon adonitol, dextrin, dulcitol, fructose, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol, starch, sucrose, or trehalose. On the other hand, the 13 soil isolates, none of which produced pyocyanin, all fermented glucose and galactose; 9, arabinose; 8, mannose; 10, xylose; and none acted upon adonitol, dextrin, dulcitol, fructose, glycerol, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol, starch, sucrose, or trehalose.

In order to observe the action of the isolates on blood, 0.5 ml of 2 per cent suspensions of washed beef corpuscles in sterile physiological saline were inoculated and incubated at 37 C for 24 hours. All of the *P. aeruginosa* group

hemolyzed the red blood cells; the *P. fluorescens* group did not. When pour plates were made with 5 per cent defibrinated beef blood agar, all the pyocyanin-producing forms showed in 24 hours at 37 C a distinct hemolysis of the beta type, with a zone of hemolysis of 1 mm or less, whereas the soil forms were characterized after 96 hours' incubation at 37 C by a slow diffuse decoloration of the hemoglobin with heavy green pigment production. When these experiments were repeated on human blood, similar results were obtained.

DISCUSSION

Evidence was produced that *Pseudomonas* sp. was isolated from cases of external otitis, and not from normal ear canals. For expressing quantitatively this association between a disease and a possible causative agent, the calculation of X^2 is frequently most advantageous (Hill, 1942). From the formula

$$X^2 = \sum \frac{(O - E)^2}{E}$$

in which O is the observed result and E the expected result, X^2 can be determined, and from that data the probability (P) of each agent being the causative one in the disease calculated.

Thus, according to the figures obtained for the frequency of each of the microorganisms isolated from both normal and diseased ears, the probability of *Pseudomonas* sp., which was isolated in 45 per cent of the pathologic cases, being the causative agent of external otitis *just by chance* is much less than 1 in 10,000; of *Staphylococcus albus*, 1 in 2,000; of fungi, 3 in 100; of diphtheroids, about 1 in 10; of viridans streptococci and *Chromobacter* sp., about 12 in 100; of hemolytic streptococci and *Sarcina lutea*, about 1 in 2; and of *Staphylococcus aureus*, about 3 in 5 (table 3). *Pseudomonas* sp. is therefore most outstanding as the probable causative agent of otitis externa. Although the value for P in the case of *Staphylococcus albus* is high, its significance decreases when the general prevalence of this organism on open surfaces and on normal tissues is taken into account.

Of foremost interest is the determination of these species of *Pseudomonas*. According to the classification of Bergey *et al.*, the 52 cultures may be grouped into the following categories: (*P. aeruginosa*, 21; class II, 9; class III, 12; class IV, 5; and class V, 5 strains. Since the activities of most of these 52 isolates differed from those species of *Pseudomonas* described by Bergey *et al.*, classes II, III, IV, and V could not be identified, but are closely related to *P. septica*, *P. schuylkilliensis*, *P. ovalis*, and *P. incognita*, respectively. Since even within a single class fermentative differences occur, each one of these categories may not contain a single species.

Further examination of the available literature disclosed that the existing data for the classification of the genus *Pseudomonas* is incomplete, and in some cases contradictory and confusing. The present definitions of the *Pseudomonas* group are therefore so inadequate as to make the proper identification of many isolates impossible.

Since all but two of the pyocyanin-producing isolates fermented arabinose,

glucose, galactose, mannose, and xylose, the fermentation of these sugars should be considered in the formation of a sound biochemical pattern for this group. This uniform ability of pyocyanin-producing bacteria to ferment arabinose, glucose, galactose, mannose, and xylose, with the formation only of acid, warrants the testing with these sugars of future *Pseudomonas* sp. isolations that are derived both from saprophytic and pathologic sources. Future data may then permit some etiologic correlation between the true natural reservoirs of pyocyanin-producing organisms and their occurrence in human pathologic processes. The irregularity of some activities, such as the reduction of nitrates to nitrites, the liquefaction of gelatin, the formation of various pigments on potato slants, and the action on litmus milk, strongly indicate the existence of different strains within this pyocyanin-producing group. Further investigations of the antigenic composition should therefore be made in an attempt to find a satisfactory serologic schema whereby each strain could be identified by its antigenic components

TABLE 3
Probability of pathogenicity of organisms

ORGANISM	X ²	P
<i>Pseudomonas</i> sp.	18.7	<.0001
<i>Staphylococcus albus</i>	12.13	.0005
Fungi	4.59	.032
Diphtheroids.	2.78	.095
Viridans streptococci	2.42	.12
<i>Chromobacter</i> sp.	2.42	.12
Hemolytic streptococci	0.51	.47
<i>Sarcina lutea</i>	0.51	.47
<i>Staphylococcus aureus</i>	0.25	.62

alone, or by its antigenic composition plus these differential biochemical and metabolic characteristics.

In addition, the pyocyanin-producing *Pseudomonas* sp. effected true beta hemolysis on blood agar plates, whereas the non-pyocyanin-producing isolates were not able to produce any degree of hemolysis. On the basis of this metabolic property, therefore, it is logical to propose that more consideration be given to these pyocyanin-producing strains as potentially etiologic agents in human disease. This characteristic is also offered as a means of differentiating in culture the pyocyanin-producing forms from those that do not produce this pigment.

Since, as a general rule, parasitic bacteria are mesophiles that grow best at the temperature of the living host, usually 37 C, the pyocyanin-formers may be considered as belonging to a potentially parasitic group. This is in direct contrast to the non-pyocyanin-formers which were psychrophilic and were isolated from saprophytic sources. The pyocyanin-producing bacteria behaved as mesophiles in that they thrived at both 42 C and 25 C, whereas their growth was markedly inhibited at 5 C. The non-pyocyanin-producing isolates behaved as

psychrophiles, as there was no evidence of stasis at 5 C and as they failed to grow at 42 C. This characteristic of different growth rates at 42 C and 5 C is offered as another difference in metabolic properties between the two groups of *Pseudomonas* strains.

SUMMARY

One hundred consecutive acute cases of external otitis were examined for the possible etiologic organisms. From 45 per cent of the patients' ears, *Pseudomonas* sp. was isolated, whereas only 16 per cent had fungi. The rest showed a variety of bacterial types. From none of the 25 normal ears were either fungi or *Pseudomonas* sp. isolated.

Studies were conducted on the biochemical and metabolic activities of 56 isolates of *Pseudomonas*, obtained from diseased ear canals, pathologic stools, well water, and soil. In general, only 6 carbohydrates of 21 tested were fermented with the formation of acid only, namely, arabinose, glucose, galactose, glycerol, mannose, and xylose. The genus was divisible into two distinct groups according to differences in (a) the production of pyocyanin, (b) the liquefaction of gelatin, (c) action in litmus milk, (d) the response to temperature extremes, (e) the production of pigment on potato slants, (f) the hemolysis of red blood corpuscles, and (g) acid formation in glycerol. These two groups could be further subdivided according to other minor variations in biochemical activity. The isolates from the soil were in a different group from those obtained from pathologic sources. It was impossible to identify the isolates specifically because of the inadequacy of the information in the available literature.

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GROWTH INHIBITION OF STRAIN H37 OF THE HUMAN TUBERCLE BACILLUS BY 4-N-ALKYLRESORCINOLS IN THE DEPTH OF A LIQUID, SYNTHETIC, NONPROTEIN CULTURE MEDIUM

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It was concluded after an earlier unreported investigation that 10^{-4} per cent of 4-*n*-hexylresorcinol in Long's liquid, synthetic, nonprotein culture medium (Long and Seibert, 1926) inhibited the depth growth of 10^{-3} mg (about 10^4 bacilli) of the H37 strain of the human tubercle bacillus.

When either *n*-hexylic acid to which equivalent molecular amounts of NaOH had been added or resorcinol was placed in the same medium, growth was prevented by 10^{-1} and permitted by 10^{-2} per cent concentrations (Drea, 1944).

It seemed desirable to investigate the antibacterial effects of other 4-*n*-alkylresorcinols.

EXPERIMENTAL

The bacteriological technique was the same as that used by the writer in 1944 for other similar studies.

The 4-*n*-alkylresorcinols were crystalline solids. Dr. L. Earle Arnow (1945) reported that all had been purified by recrystallization, except the ethyl, propyl, and butyl compounds, which were prepared by distillation only. All of them were dissolved in sterile solutions of 30 per cent glycerol in distilled water and placed in a dark closet at room temperature. The following percentages of concentrations—2 and 2×10^{-1} of ethyl, propyl, butyl, and amyl; 10^{-1} of hexyl and heptyl; and 2×10^{-2} of octyl, decyl, undecyl, dodecyl, and tetradecyl resorcinols—were sterile at the end of 24 hours. Any clouded suspensions were made clear by warming them. The foregoing concentrations were then diluted with sterile distilled water to give 2×10^{-1} , 2×10^{-2} , etc., per cent concentrations, and from these 1-ml amounts were taken and dropped into 19 ml of Long's liquid synthetic culture medium to give 10^{-2} , 10^{-3} , etc., per cent concentrations of the substances to be tested for their antibacterial effects. Smaller gradations than decimal dilutions were not studied.

A check was made for possible additive antibacterial effects of both hexylic and heptylic soaps separately on that of resorcinol. Equivalent molecular amounts of NaOH were added to the fatty acids (from Eastman Kodak Company). The three substances were prepared as sterile solutions in the same way as were the alkylresorcinols. The proper amounts of either fatty acid compound and resorcinol were placed in the culture medium to give the desired equivalent total weight of the corresponding alkylresorcinol.

Plantings of 10^{-3} mg of well-dispersed H37 bacilli were made and incubated

at 37 C for at least 60 days. Evaporation of water was nil. Three separate growth experiments were made for each tested substance with the exception of the hexyl and heptyl resorcinols for which 5 and 4 tests, respectively, were made. The pH of the culture media was 7.2.

RESULTS

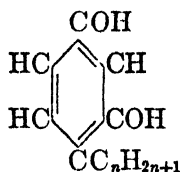
Seven of the 11 alkylresorcinols inhibited growth at 10^{-4} per cent concentrations. All of these had 6 or more carbon atoms in the alkyl chain. The hexylresorcinol permitted questionable visible growth in three out of five trials and no growth in the other two attempts. The heptylresorcinol allowed one small clump of bacilli to grow in one out of four trials, the other three permitting no growth.

Ethyl, butyl, and amyl resorcinols with 2-, 4-, and 5-carbon atom side chains, respectively, inhibited growth at 10^{-3} per cent concentrations. The propyl resorcinol with a 3-carbon atom chain inhibited growth at 10^{-2} per cent concentrations. The mixture of either hexylate or heptylate with resorcinol permitted growth at 10^{-2} and inhibited at 10^{-1} per cent concentrations.

Only growth-inhibiting powers for 60-day incubation periods are reported. It was established that incubation for some time longer than 60 days or transplanting to fresh Long's liquid medium at the end of 60 days permitted growth of previously invisible cultures or plantings in 10^{-4} per cent *n*-hexylresorcinol media.

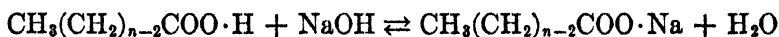
DISCUSSION

The 4-*n*-alkylresorcinols are produced by condensing resorcinol with the proper fatty acids in the presence of ZnCl_2 , followed by reduction according to the method of Clemmensen (Suter, 1941). The alkyl chain $\text{C}_n\text{H}_{2n+1}$ is attached to the carbon atom in the 4-position of the resorcinol molecule as represented by:



If *n* has the value of 6, 7, 8, 10, 11, 12, or 14, the growth-inhibiting amount is of order of 10^{-4} per cent. Growth is permitted by 10^{-5} per cent.

The reaction between a fatty acid and NaOH in water may be represented by the reversible formula:



Here, *n* must have the value of 14 or 16 for the 14- and 16-carbon atom chains of myristic and palmitic acids, respectively, in order to inhibit growth at 10^{-4} per cent under the same conditions (Drea, 1944).

The hexylic and heptylic acids with 6- and 7-carbon chains, respectively, have considerably less antibacterial power. The substitution, however, of the hexyl

or heptyl chain in the 4-position of the weakly inhibiting resorcinol ring results in a molecule with considerably greater antibacterial power.

The ethyl, propyl, butyl, and amyl resorcinols have less inhibiting power than the hexyl and heptyl resorcinols, but are also characterized by being more growth-inhibiting than their side chains as fatty acids and soaps or resorcinol. The lesser antibacterial property of the propyl resorcinol as compared with that of the ethyl and the butyl compound may be due to the fact that none of the three compounds were recrystallized.

It was indicated that the octyl and longer alkyl chain resorcinols tested were somewhat more antibacterial than the hexyl and heptyl resorcinols, but within

TABLE 1
Effect of alkylresorcinols on the growth of tubercle bacilli
10⁻⁸ mg of well-dispersed H37 bacilli planted
Incubation for 60 days at 37 C

NUMBER OF C ATOMS IN ALKYL CHAIN	TYPE OF ALKYLRE- SORCINOL TESTED	GROWTH-INHIBITING CONCENTRATION		GROWTH- PERMITTING CONCENTRATION PER CENT
		Number of tests	Per cent	
2	Ethyl	2 out of 3	10 ⁻³	10 ⁻⁴
		1 out of 3 with very small amount of growth	10 ⁻³	
3	Propyl	3 out of 3	10 ⁻²	10 ⁻³
4	Butyl	3 out of 3	10 ⁻³	10 ⁻⁴
5	Amyl	3 out of 3	10 ⁻³	10 ⁻⁴
6	Hexyl	2 out of 5	10 ⁻⁴	10 ⁻⁵
		3 out of 5 with questionable growth	10 ⁻⁴	
7	Heptyl	3 out of 4	10 ⁻⁴	10 ⁻⁵
		1 out of 4 with very small amount of growth	10 ⁻⁴	
8	Octyl	3 out of 3	10 ⁻⁴	10 ⁻⁵
10	Decyl	3 out of 3	10 ⁻⁴	10 ⁻⁵
11	Undecyl	3 out of 3	10 ⁻⁴	10 ⁻⁵
12	Dodecyl	3 out of 3	10 ⁻⁴	10 ⁻⁵
14	Tetradecyl	3 out of 3	10 ⁻⁴	10 ⁻⁵

the limits of error of this investigation the longest chain compound, tetra-decyl-resorcinol, had the same order of antibacterial power as the 14-carbon atom chain of myristic acid.

The antibacterial effect of organic compounds with long carbon chains continues to excite interest in tuberculosis research. Hook and Robinson (1944) are investigating synthesized derivatives of dialkylbutyric acids. Roberts (1945) is studying agaric acid and related compounds.

Barry and McNally (1945) concluded from their investigation of dialkyl succinic acid derivatives that *a*-ethyl-*a'*-dodecyl succinic monomethyl ester inhibited growth of a bovine strain *in vitro* for 6 weeks at 1:400,000, and that the isomeric *a*-*a'*-di-*n*-heptyl acid ester inhibited for 6 weeks at 1:300,000. They

were of the opinion that it was indicated that the molecular weight of the acid, or, alternately, the total number of carbon atoms in the two substituents, must lie within a definite range to produce maximum tuberculocidal activity in the half-ester.

Fatty acids with fewer carbon atoms may also be of importance, because, when condensed with weakly inhibiting molecules such as those of resorcinol, they acquire a much greater growth-preventing action *in vitro* on the H37 strain of the tubercle bacillus.

The present writer, in 1944, suggested that the addition of a 6-carbon atom or longer alkyl chain at the 4-position of the greatly inhibiting sodium ethylmercuri-thiosalicylate molecule, with or without the substitution of some element such as Cd or Mn for the Hg atom, might be advantageous. It may be desirable to add the 2- to 5-carbon atom chains in the same way.

SUMMARY

Hexylic acid and heptylic acid, to which equivalent molecular amounts of NaOH have been added, prevent the growth of 10^{-8} mg (about 10^8 bacilli) of the H37 strain of the human tubercle bacillus in the depth of Long's liquid, synthetic, nonprotein culture medium when present in 10^{-1} per cent concentrations and permit growth in 10^{-2} per cent concentrations. Resorcinol under the same conditions has the same effect.

The corresponding *n*-alkylresorcinols with the hexyl and heptyl chains substituted for the H atom in the 4-positions of the resorcinol molecules inhibit growth at 10^{-4} per cent and permit growth at 10^{-5} per cent concentrations.

Octyl, decyl, undecyl, dodecyl, and tetradecyl chain resorcinols have the same order of growth-preventing power. The first four of these are more inhibiting than the corresponding fatty acids or resorcinol. Tetradecyl resorcinol has about the same growth-preventing effect as the corresponding myristic acid.

Ethyl, propyl, butyl, and amyl resorcinols have less growth-inhibiting power than the longer chain compounds but are more inhibiting than their respective fatty acids or resorcinol.

The addition of alkyl chains to the 4-position of the greatly inhibiting sodium ethylmercuri-thiosalicylate molecule, with or without the substitution of some other element for the Hg atom, may be of antibacterial importance in both *in vitro* and chemotherapeutic studies.

Bactericidal effects were not studied.

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THE CECAL FLORA OF WHITE RATS ON A PURIFIED DIET AND ITS MODIFICATION BY SUCCINYLSULFATHIAZOLE¹

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Comparatively few types of microorganisms appear able to establish themselves in the intestinal tract of man and the lower animals. However, the relative proportions of these types may be influenced by the ingestion of certain bacterial species, by dietary modifications, or by the administration of drugs. This alterable character of the intestinal microflora was known to Metchnikoff (1907) who used sour milk therapy to establish lactic acid bacilli in the intestinal tube. Successful transformation of the intestinal flora to an aciduric type by the feeding of *Lactobacillus acidophilus* was demonstrated by Rettger and Cheplin (1921). Extensive studies (Rettger and Horton, 1914; Hull and Rettger, 1917; Herter and Kendall, 1909; Torrey, 1919; Winblad, 1941; Porter and Rettger, 1940; Mitchell and Isbell, 1942) have shown that the intestinal flora is affected by the kind of food ingested; that a predominance of proteolytic (or gram-negative) bacteria may be induced when the diet consists largely of meat or other protein food; and that saccharolytic (or gram-positive) organisms become dominant when the diet is high in carbohydrates. It has been claimed that lactose and dextrin are more effective in producing this modification than are other carbohydrates. Likewise milk, presumably because of its lactose content, appears to favor the establishment of an acidophilic flora.

The early use of drugs as the modifying agent, initiated by Bouchard (1887), was intended to inhibit the putrefactive processes of organisms of the gastrointestinal tract. The results were for the most part disappointing. In modern usage, the sulfonamides have proved exceedingly valuable, both clinically and in nutritional experiments, in the simplification of the intestinal flora (Marshall *et al.*, 1940; White, 1942; Welch *et al.*, 1942; Poth *et al.*, 1942; Gant *et al.*, 1943; Miller, 1945).

Until recent years, studies of the intestinal organisms were made to determine whether their presence was purely incidental or whether their putrefactive processes could be adverse to the well-being of the host. The present tendency is to shift emphasis from the viewpoint that intestinal bacteria are saprophytes whose metabolic products might be harmful, to a consideration of them as important to the well-being of the animal through their ability to synthesize essential substances. Evidence supporting this concept has been sought through the use of sulfonamides which have only a slight toxicity, which are but slowly absorbed from the intestinal tract, and which modify the flora by inhibiting the development of certain groups of organisms.

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Following the indications from the work of Black *et al.* (1941), Gant *et al.* (1943) made the first extensive attempt to correlate changes occurring in the intestinal flora with the reduced rate of growth or the occurrence of symptoms of vitamin deficiency in rats receiving a purified diet to which sulfonamides were added. This paper represents a continuation of that study and purposes (1) to outline a comparatively simple method by which a fairly comprehensive picture of the numerically important groups of the intestinal microflora may be obtained; (2) to report the results of the practical application of this method to a study of the cecal flora of white rats on the purified diet of Black *et al.* (1941); and (3) to record in detail the modifying effects on the cecal flora produced by the inclusion of succinylsulfathiazole in the diet. This drug was selected after comparative studies with sulfathiazole, sulfamerazine, and sulfadiazine indicated its greater efficacy and lesser toxicity to the rat. Cecal bacteria only were investigated, since recent evidence from cecectomy (Guerrant *et al.*, 1935; Taylor *et al.*, 1942; Day *et al.*, 1943) pointed toward the particular importance of the cecum in vitamin synthesis.

MEDIA

The judicious choice of media is a highly important factor in an investigation of the type reported. It is essential that one medium give as nearly as possible a "total count" of the viable bacterial population and that selective media be used which permit estimation of the relative numbers of each of the main bacterial groups. In the course of the development of methods, a number of selective media were tried and subsequently discarded when their inadequacies became apparent. Inclusion of a medium in the series depended primarily upon proof of its selective capacity and secondarily upon ease of preparation and practicability in the reading of results.

Liver infusion broth, composed of bacto-tryptone 5 g, glucose 5 g, liver extract 100 ml, tap water to 1,000 ml, and tubed with added dried liver chunks, was found satisfactory for estimating the "total numbers" of viable bacteria because it offers an adequate nitrogen and carbon nutrient complex for the diverse intestinal types. (The liver extract was made by steaming 1 pound of liver in 2 liters of tap water and filtering through gauze.) The reducing action of the liver tissue and the use of a deep column of medium promote growth of anaerobic as well as aerobic bacteria. The only intestinal group for which it is known to be unsatisfactory is the yeastlike microorganisms which occurred frequently in the cecal contents and which, in certain instances, have been found to dominate the flora. For these a special medium, referred to later, is required.

The estimation of the coliform group presents a difficult problem when they are outnumbered by other forms, as it was known they would be in the drug-treated animals. E. M. B. agar and several varieties of lactose broth were tested, but, in most cases, the results were not sufficiently clear-cut or conclusive, indicating that none of these media was selective enough for isolation of this group from the varied population existing in the cecum. The EC medium of Hajna and Perry (1943) proved the most satisfactory and appeared to justify the authors' claims of selective culture of the coliform group and more or less com-

plete inhibition of fecal streptococci, by virtue of the added bacto bile salts no. 3. Two series of this medium were inoculated: one, incubated at 37 C, was intended to detect the coliform bacteria as a group; the other, incubated at 45 C, was used for the *Escherichia coli* determination. Readings on the basis of gas production were made by the dilution count method.

A specific medium for the enumeration of the enterococci was found in the SF medium of Hajna and Perry (1943), which utilizes NaN_3 (0.5 g per L) as its inhibitory agent and a selective temperature of incubation (45 C). Turbidity and an acid reaction shown by the color change of the bromcresol purple indicator were the criteria of growth.

Winblad's (1941) acetic acid medium, minus the agar, was selected for the estimation of the numbers of lactobacilli after its efficiency had been tested against Kulp's tomato juice medium (with and without agar). Because of the highly satisfactory results of this comparison and the greater simplicity in its preparation, this acetic acid medium was accepted in the routine. Later observation showed that yeasts also grew well in this medium and evolved an abundance of gas which could be trapped in Durham tubes and easily detected if the test tubes were vigorously shaken at intervals during the incubation period. The depth of this medium in the test tube was therefore practically equivalent to the enclosed fermentation tube. This medium with incubation at 37 C then served for the determination of the numbers of both lactobacilli and yeasts. However, since the yeast count might exceed that of the lactobacilli; since the turbidity due to lactobacillus growth might be rather slight; since a certain amount of sediment in the medium made readings somewhat difficult; and since, in the absence of lactobacilli, enterococci appeared in this medium, the results were routinely checked after 72 to 96 hours of incubation by microscopic examination and by subcultures on blood agar plates made in duplicate for incubation under aerobic and anaerobic conditions. The fact that incubation temperature of 10 C inhibits growth of lactobacilli, yet permits enterococci to proliferate, offered a check on the reliability of the SF medium and was so used in the early stages of this experiment. Comparison of enterococcus growth in the acetic acid medium incubated at 10 C with that in SF medium incubated at 45 C showed good agreement.

METHODS

Weanling albino rats from the Sprague-Dawley colony were fed the purified basal ration of Black, McKibbin, and Elvehjem (1941) which consisted of:

Sucrose.....	76 g
Purified casein.....	18 g
Salts.....	4 g
Corn oil.....	2 g
Choline chloride.....	200 mg
Nicotinic acid.....	2.5 mg
Ca-pantothenate.....	2.0 mg
Pyridoxine.....	0.3 mg
Thiamine.....	0.3 mg
Riboflavin.....	0.3 mg

Each rat received also 2 drops of haliver oil per week. One group of rats received only this ration, another received this ration containing 0.5 per cent succinylsulfathiazole.

At intervals of 5, 10, 15, 21, and 28 days, animals from each group were sacrificed, and bacterial counts were made according to the procedure outlined below.

Under aseptic conditions each animal was opened, and 0.1 ml of cecal contents was removed with a blunt end pipette and was transferred to a 10-ml water blank bottle containing a few glass beads to facilitate uniform suspension. Following vigorous and thorough shaking (routinely 200 times), this suspension (10^{-2}) of material was further diluted in decimal series to 10^{-11} . By means of quantitative inoculations of 1 ml for the lower ranges and of 0.25, 0.5, 0.75, or 1 ml for the higher, it was possible to estimate growth in the test tubes of media at dilutions of 10^{-3} to 10^{-9} and $10^{-9.25}$, $10^{-9.5}$, $10^{-9.75}$, 10^{-10} , $10^{-10.25}$, $10^{-10.5}$, $10^{-10.75}$, and 10^{-11} . Obviously such a series of dilutions eliminates the disproportionate spread between the decimal dilutions, 10^{-9} (1,000,000,000) and 10^{-10} (10,000,000,000) and between 10^{-10} (10,000,000,000) and 10^{-11} (100,000,000,000) and thus allows more precision in the dilution count data.

Triplicate inoculations were made for each series of media. The inoculated tubes were incubated at 37 C or 45 C, as needed, for a period of 72 hours, after which readings were made on the basis of turbidity, acid production, or the evolution of gas, and checked by smear and by transfer to petri plates containing appropriate media.

Studies made by wet preparations of material either directly from the cecum or from the 10^{-2} dilution, to determine whether or not protozoan parasites might have become established, failed to reveal microorganisms other than bacteria and yeastlike organisms. This absence of microfauna, anticipated in the Sprague-Dawley animals, may be significant since this investigation is concerned only with the microflora involved alone or symbiotically in the intestinal synthesis of vitamins.

During the early part of the experiment, when the method was on trial, direct bacterial counts on smears from the 10^{-2} dilutions, stained according to the method of Gram, were used as a check on the accuracy and adequacy of the cultural counts. The percentage of gram-positive rods, gram-negative rods, streptococci, micrococci, fusiform bacilli, vibrios, spirochetes, bacterial spores, and mold spores and mycelium were calculated from a count of 300 cells.

RESULTS

The averages of the counts obtained for each series of animals are recorded in table 1. The figures for the "total counts" were for the most part consistent; those for certain of the groups, particularly the enterococci and the yeastlike organisms, fluctuated. Table 2 and table 3 are included to show the individual animal data for the "total counts" and for the enterococcus count.

DISCUSSION

Profound changes in the intestinal microflora have been observed in various nutritional and clinical investigations involving the use of succinylsulfathiazole.

The phenomenal reduction in numbers of the coliform group has been consistently reported (Marshall *et al.*, 1940; White, 1942; Welch *et al.*, 1942; Poth *et al.*, 1942; Gant *et al.*, 1943; Miller, 1945). In nutritional experiments interest has

TABLE 1
The cecal flora of white rats

DAYS ON DIET	NO. OF ANIMALS	TOTAL AND GROUP COUNTS—AVERAGES FROM DILUTION COUNT DATA					
		Total count	Lactobacilli	Enterococci	Coliforms (37 C)	<i>E. coli</i> (45 C)	Yeastlike organisms
5	3 Controls	25,000,000,000	5,000,000,000	25,000,000	2,500,000,000	1,000,000	25,000,000
	3 Drug-treated	10,000,000,000	5,000,000,000	25,000,000	50,000	10,000	1,000
10	3 Controls	25,000,000,000	10,000,000,000	5,000,000	2,500,000,000	50,000,000	5,000
	3 Drug-treated	50,000,000,000	25,000,000,000	50,000,000	2,500,000	250,000	100,000,000
15	3 Controls	7,500,000,000	5,000,000,000	50,000,000	1,000,000,000	750,000,000	1,000,000
	3 Drug-treated	10,000,000,000	100,000,000	2,500,000,000	1,000	1,000	10,000,000
21	3 Controls	10,000,000,000	2,500,000,000	50,000,000	1,000,000,000	50,000,000	10,000
	3 Drug-treated	2,500,000,000	50,000,000	1,000,000,000	2,500,000	250,000	2,500,000
28	3 Controls	7,500,000,000	5,000,000,000	10,000	7,500,000	1,000,000	100,000
	3 Drug-treated	2,500,000,000	50,000,000	500,000,000	5,000,000	5,000,000	1,000,000

TABLE 2
The range of total counts as estimated from liver infusion medium

DAYS ON DIET	NO. OF ANIMALS PER GROUP	CONTROL ANIMALS	DRUG-TREATED ANIMALS
5	3	50,000,000,000	10,000,000,000
		5,000,000,000	25,000,000,000
		10,000,000,000	10,000,000,000
10	3	50,000,000,000	50,000,000,000
		5,000,000,000	75,000,000,000
		10,000,000,000	25,000,000,000
15	3	25,000,000,000	10,000,000,000
		5,000,000,000	10,000,000,000
		5,000,000,000	10,000,000,000
21	3	25,000,000,000	7,500,000,000
		7,500,000,000	100,000,000
		5,000,000,000	500,000,000
28	3	10,000,000,000	5,000,000,000
		5,000,000,000	1,000,000,000
		5,000,000,000	1,000,000,000

centered on this group and particularly on *E. coli*, since Dam *et al.* (1941) directed attention to its probable importance in vitamin synthesis. Clinically, as Poth points out, quantitative determination of the coliform group is readily

accomplished and offers a satisfactory means of following the alterations occurring in the flora. The striking depressant effect of succinylsulfathiazole on the coliform group was evident in this study also. The decrease was demonstrable in 5 days and was greatest at 15 days; thereafter an upward trend was manifest. The coliform group, which, in the normal animals, comprised 10 to 25 per cent of the total number of 7,500,000,000 to 25,000,000,000 organisms per ml of cecal contents, dropped to 1,000 or less in the drug-treated animals. No coincidental decrease in total numbers of bacteria accompanied this change in coliform numbers. On the contrary, there appeared at the 10-day sampling an actual increase in population, which, at the 21- and 28-day intervals, gave way to a

TABLE 3
The range of enterococcus counts as estimated from SF medium

DAYS ON DIET	NO. OF ANIMALS PER GROUP	CONTROL ANIMALS	DRUG-TREATED ANIMALS
5	3	100,000,000	100,000,000
		1,000,000	1,000,000
		1,000	<1,000
10	3	10,000,000	100,000,000
		1,000,000	10,000,000
		1,000,000	<1,000
15	3	100,000,000	5,000,000,000
		100,000	5,000,000,000
		<1,000	1,000,000,000
21	3	100,000,000	2,500,000,000
		50,000,000	1,000,000,000
		5,000,000	1,000,000,000
28	3	100,000	1,000,000,000
		1,000	100,000,000
		<1,000	100,000,000

marked reduction from the original total numbers. Coliform species other than *E. coli* appeared to dominate consistently (in a ratio roughly of 10:1) and to be susceptible to the effects of the drug. These results on the gram-negative group of organisms agree with the findings of Gant *et al.* (1943) and Miller (1945), both of whom utilized the same or similar diets for their animals. However, no significant decrease in the "total count" was reported by these observers as was noted at the 21- and 28-day sampling in this study.

Numerically, the most important microorganisms in the cecal contents of the control animals (those receiving the purified diet only) were the lactobacilli which constitute 25 to 75 per cent of the "total numbers" of organisms. Succinylsulfathiazole exerted a depressing effect on this group also, but the reduction in numbers was slower and less drastic than that of the coliform bacteria and was

maintained up to the end of the experiment. This effect of the drug has not been previously reported, and its significance is as yet unknown. However, Poth (1942) describes a marked reduction (10^{11} to 10^5) of the gram-positive bacteria in certain clinical cases. Further mention of the effect of succinylsulfathiazole on the gram-positive organisms in the human intestinal tract has been made by Lockwood (1942), who observed a drop in the number of clostridia.

Two groups of organisms, the fecal streptococci and certain yeastlike organisms, were not only not harmed by the drug but apparently increased in the drug-treated animals.

The extent to which sporeforming microorganisms exist in the cecum was not investigated. Miller (1945) estimated the anaerobic spore numbers at 10^3 per g of wet feces, a figure not changed by the addition of succinylsulfathiazole. Gant *et al.* (1943) observed spores to a maximum of 10^3 per ml of cecal contents in approximately one-half of the rats on the purified diet. In view of these evidences of low concentration, and the fact that a spore count is not an estimate of the actual numbers of sporeforming organisms in the population, spore counts in the present study were deemed nonessential.

The results of this investigation indicate that the purified diet of Black *et al.* (1941), with its high sucrose content, favors the development of a mixed microflora in which acidophilic types predominate.

The total viable count of organisms in the cecum was found to be high ($10^{9.75}$ to $10^{10.5}$ per ml). The counts obtained by Gant *et al.* (1943) are similar (10^{10} to 10^{11} per ml), whereas those obtained by Miller (1945) are lower (10^8 for aerobes, 10^7 to 10^8 for anaerobes), a fact explainable on the basis of the method employed, since lactose broth was used by Miller for the estimation of aerobes and thio-glycollate medium for the anaerobes.

The sum of the group counts obtained in the present study comprised about 1/3 to 2/3 of the apparent "total" of organisms. The fact that the same ratio held for both controls and drug-treated animals would indicate that there was no great overlap in the selective counts, a good point when one wishes to consider absolute figures as in coliform and lactobacillus reductions or enterococcus and yeast increases. The discrepancy between "total" count and the sum of the groups might be attributed to the presence of an uncounted group, such as the bacteroides, which have received scant attention by bacteriologists in general because they are difficult to handle and because their physiology is unknown. Study of the bacteroides in relation to the intestinal flora in dietary experiments must wait upon the development of methods.

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SUMMARY

In a study of the cecal flora of albino rats on the purified diet of Black *et al.*, the largest group counted was found to be the lactobacilli. The coliform group was also present in important numbers and included in large proportion coliform species other than true *E. coli*. Enterococci and yeastlike organisms were less numerous than either the lactobacilli or the coliform bacteria. "Total" counts always exceeded the sum of the group counts, indicating the presence of types, perhaps bacteroides, for which no group count method is yet available.

The feeding of a diet containing 0.5 per cent succinylsulfathiazole depressed the coliform and the lactobacillus groups. The former, although more sharply affected, showed a tendency toward re-establishment; the latter showed a slower alteration in numbers but a more permanent one. The reduction in numbers of these two groups was in part compensated by the increase in numbers of enterococci and yeastlike forms.

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THE SEGREGATION OF ANTIGENS IN A BACTERIAL CULTURE BY AN UNDESCRIBED FORM OF VARIATION¹

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In November, 1943, an unidentified *Salmonella* culture (N153) was received from Major K. S. Wilcox. The organism, which was isolated by Major P. R. Carlquist from a case of gastroenteritis in Exeter, England, was said to contain XXIX and Vi antigens. This statement was confirmed, and, in addition, the bacillus was found to have H antigens unlike any previously encountered by the writer.

In December, 1943, Dr. J. J. Monteverde sent a culture of his new type, *Salmonella hormaechei*, the antigens of which were designated as XXIX:z₃₀ (Monteverde, 1944).

Because of a misunderstanding a second culture of *S. hormaechei* was forwarded from Dr. Monteverde's laboratory, and, to distinguish the two, the first culture was labeled 1 and the second, 4. Upon inquiry it was found that the two transplants were derived from the same strain, which was isolated from the ovary of a hen. Transplant 1 was said to be the result of "300 selections," but 4 represented an "unselected" culture from the parent stock. It was stated that neither of the cultures was exposed to the action of agglutinating serum.

Later Dr. Monteverde sent a third culture (LC54), which was isolated from sewage. The formula of this culture was given as XXIX [Vi]:z₃₀ (Monteverde, 1944; Monteverde and Leiguarda, 1944), and it was further stated that through induced variation brought about by the technique of Gard (1937) the H antigens of this culture could be changed to z₁₄, i.e., the H antigens of *Salmonella ballerup*. This z₁₄ ⇌ z₃₀ variation was named "zeta variation." Later, as the result of continued work with cultures LC54 and N153, Monteverde (1945) postulated another antigen, z₃₁, in these cultures as well as in *S. ballerup*.

Up to the present time not all the observations of Monteverde have been confirmed in this laboratory, and since some of these matters are still in an unsettled state they will not be discussed at length. The object of this communication is simply to set forth the results obtained in the study of the H antigens of N153, 1, 4, and LC54, and to describe an unusual variation which occurred in the H antigens of LC54.

It should be emphasized at the outset that the work set forth deals only with the H antigens of the cultures. The O antigens of the cultures were all XXIX [Vi], and no changes were noted in them except V-W variation, which affected only the Vi content of individual colonies and their progeny. All the tests were

¹ The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

performed with serums from which somatic agglutinins had been removed. The motility of some of the cultures was rather slight, so that it was necessary to pass them repeatedly through semisolid agar to secure cultures which were actively motile and which flocculated rapidly with appropriate serums.

The results obtained in the study of the H antigens are given in table 1. *S. hormaechei* 4 and N153 are of similar antigenic structure and differ from *S. hormaechei* 1. A unilateral relationship exists between *S. hormaechei* 1 and N153 in which the former is flocculated in high dilution in N153 serum, but N153 is agglutinated little, if at all, by *S. hormaechei* 1 serum. Such one-sided relationships are found among strains of *Shigella* (Wheeler, 1944), the colon bacilli (Kauffmann, 1944), and, to a less pronounced degree, among other *Salmonella* strains. That this discrepancy is not due to the response of individual rabbits to these antigens is indicated by the fact that two rabbits were injected with each culture and all the serums gave reactions similar to those cited in table 1. It can be seen that *S. hormaechei* 1 is quite distinct from *S. hormaechei* 4 and N153, yet 1 and 4 are supposedly descendants of the same culture. In observations extending over 2 years no changes were noted in the cultures which would indicate that the differences observed were due to simple phase variation. Apparently the cultures are distinct antigenic types which are fixed under ordinary conditions of culture.

Culture LC54 is agglutinated to titer by *S. hormaechei* 1 and N153 serums, and likewise LC54 serum agglutinates both *S. hormaechei* 1 and N153 in high dilution. In absorption tests LC54 removes all agglutinins from *S. hormaechei* 1 serum as well as from N153 serum, indicating that LC54 has all the antigens of both types. When LC54 serum is absorbed by *S. hormaechei* 1 agglutinins active against N153, *S. hormaechei* 4 and LC54 remain in the serum. If it is absorbed by *S. hormaechei* 4 or N153, it is still active against *S. hormaechei* 1 and LC54. However, if LC54 serum is absorbed by *S. hormaechei* 1 and N153 in combination, all agglutinins are removed from the serum. These tests indicate that LC54 contains all the principal antigens present in N153 and *S. hormaechei* 1 but that it contains no other major components. According to the method which Monteverde (1945) has used in designating the antigens of these cultures, their major antigenic components would be expressed as follows:

<i>S. hormaechei</i> 4 and N153	— XXIX [Vi]: z_{30} ...
<i>S. hormaechei</i> 1	— XXXIX [Vi]: z_{31} ...
<i>S. hormaechei</i> LC54	— XXIX [Vi]: z_{30} , z_{31}

When LC54 was first received, it was plated and two sorts of colonies, which were distinguished only by their H antigens, were found. The first resembled N153 and *S. hormaechei* 4 (z_{30}). This variant was labeled LC54A. On further culture and plating these colonies have remained constant for 2 years, and no variation was noted in the examination of more than 400 colonies. In addition, serums prepared from strains that had been isolated for more than a year gave no evidence of change in the cultures. The second type of colony resembled the parent culture (z_{30} , z_{31}). On serial plating these colonies continued to produce colonies about 5 per cent of which contained only z_{30} and 95 per cent of which

contained z_{30} , z_{31} . At this point the work was interrupted, and the cultures in question were placed in tightly stoppered tubes of semisolid agar and kept at room temperature for approximately 1 year.

TABLE 1
Agglutination reactions

SERUMS		ANTIGENS					
		1	4	N153	LC54	LC54A	LC54B
1	Unabsorbed	10,000	0	0	10,000	0	10,000
	Absorbed by						
	4 or N153	10,000	0	0	10,000	0	10,000
	LC54	0	0	0	0	0	0
	LC54A	10,000	0	0	10,000	0	10,000
	LC54B	0	0	0	0	0	0
N153	Unabsorbed	5,000	10,000	10,000	10,000	10,000	0
	Absorbed by						
	1	0	5,000	5,000	5,000	5,000	0
	4	0	0	0	0	0	0
	LC54	0	0	0	0	0	0
	LC54A	0	0	0	0	0	0
	LC54B	5,000	5,000	5,000	5,000	5,000	0
LC54	Unabsorbed	10,000	5,000	5,000	10,000	5,000	10,000
	Absorbed by						
	1	0	5,000	5,000	5,000	5,000	0
	4 or N153	10,000	0	0	10,000	0	10,000
	1 + N153	0	0	0	0	0	0
	LC54A	10,000	0	0	10,000	0	10,000
	LC54B	1,000	5,000	5,000	5,000	5,000	0
	LC54A + LC54B	0	0	0	0	0	0
LC54A	Unabsorbed	2,000	10,000	10,000	5,000	10,000	0
	Absorbed by						
	1	0	5,000	5,000	5,000	5,000	0
	4 or N153	0	0	0	0	0	0
	LC54	0	0	0	0	0	0
	LC54B	2,000	5,000	5,000	5,000	5,000	0
LC54B	Unabsorbed	10,000	0	0	10,000	0	10,000
	Absorbed by						
	1	0	0	0	0	0	0
	4 or N153	10,000	0	0	10,000	0	10,000
	LC54	0	0	0	0	0	0
	LC54A	10,000	0	0	10,000	0	10,000

Figures indicate the highest dilution at which agglutination occurred; 0 indicates no agglutination at 1 to 200.

When the work was resumed, the z_{30} cultures were found unchanged, but from the z_{30} , z_{31} forms no more similar loss variants could be obtained. More than 800 colonies of various z_{30} , z_{31} substrains were examined, and no z_{30} colonies were

found. On the contrary, approximately 12 per cent of the colonies contained only z_{31} . This variant was labeled LC54B. These colonies, which were similar to, but not identical with, *S. hormaechei* 1, continued to appear in cultures that were plated serially 8 times from well-isolated single colonies and were still present when the cultures were last examined. Like the z_{30} variants, these z_{31} variants were quite stable. More than 300 colonies were examined over a period of 1 year, and no evidence of change was observed. Likewise serums prepared from the colonies contained agglutinins only for the major antigenic component.

The characteristics of the parent culture (z_{30} , z_{31}) and z_{30} and z_{31} variants are also included in table 1. The reactions of LC54A (z_{30}) exactly paralleled those of N153 and *S. hormaechei* 4, and since LC54A and N153 are mutually absorptive they may be considered identical in major antigenic components. *S. hormaechei* 1 and LC54B are also mutually absorptive and, therefore, presumably the same. Yet the two cultures react in an entirely different manner in N153 and LC54A serums. *S. hormaechei* is flocculated in high dilution by these serums, but LC54B is agglutinated little, if at all, by them. Further, although LC54B removes all agglutinins from *S. hormaechei* 1 serum, it will not remove agglutinins for *S. hormaechei* 1 from N153, LC54, or LC54A serums. These contradictory reactions were repeated many times using different antigen suspensions, and the results were invariably the same.

As would be expected, LC54 (z_{30} , z_{31}) and all the single colony isolations which resembled the parent culture were capable of removing agglutinins from both LC54A (z_{30}) and LC54B (z_{31}) serums. Neither of the substrains was capable of removing agglutinins from LC54 serum, each leaving a large residue of agglutinins for the parent culture and for the other variant. If LC54A and LC54B were used in combination, they absorbed all agglutinins from the serum of the parent strain. From these results it is apparent that the variants, LC54A and LC54B, contain no major H components in common, but added together they contain all the major antigens of the culture from which they were derived.

DISCUSSION

The discrepancies noted in the serological behavior of *S. hormaechei* 1 and LC54A are difficult to explain. It might be said that varying amounts of a minor antigen in the various cultures accounted for the contradictory results, but that hypothesis would not explain all the observations. From the results obtained one is forced to conclude that cultures which are mutually absorptive are not always identical in their serological behavior. Similar discrepancies have been noted in the serological study of *Escherichia coli* (Kauffmann, 1945). It must be remembered that in this instance loss variants are being studied and some irregularities may be expected. Although these apparent discrepancies detract from the regularity of the results, they by no means invalidate them. The important fact remains that a complex culture is capable of dissociating two loss variants and that these loss variants are similar to cultures found in nature.

The possibility that LC54 was a mixed culture must be considered. All of the observations speak against this possibility. When the culture was first

received, it was plated and isolations made from single colonies. Further, the variation was not eliminated by serial plating; the variants continued to appear in cultures repeatedly picked from well-isolated single colonies. In addition, it seems significant that the two variants were not found simultaneously but appeared at different periods. Variant A was isolated many times without difficulty when the culture was first received, but variant B appeared only after variant A could no longer be found. It would appear that the culture dissociated in one way at a given time, but in an entirely different manner at another time. Also, it is extremely unlikely that three such unusual forms as LC54, LC54A, and LC54B, all having the same O antigen and Vi antigen, and the H relationships shown by the three, should coexist in a single culture. Finally, and probably most conclusive, are the results obtained by a modification of the method of Gard (1937). LC54A was inoculated into tubes of semisolid agar containing its corresponding antiserum which had been freed of O agglutinins. Only after several serial transfers in this medium did the culture show evidence of variation by migrating through the medium. From the spreading growth was isolated a form identical with LC54B as judged by agglutination and absorption tests. By prolonged growth and serial transfers in medium containing LC54B serum, single colonies of this form were again reverted to the original state, i.e., a culture indistinguishable from LC54A. The fact that one of the variants could be transformed to the other and then reverted to its original state by induced variation is strong evidence that both arose from a common parent and not from a mixed culture.

It is obviously impossible to state definitely that the loss variants recovered from LC54 are stable and never revert to the parent type. It can only be said that under the conditions of the experiments no variation was noted in the H antigens of either variant. Variant A has been observed for 2 years and variant B for 1 year. Hundreds of colonies of each were examined, and serums were prepared for both. No evidence of reversion of A (z_{30}) or B (z_{31}) to the parent type (z_{30} , z_{31}) was noted. Induced variation by serums could not be used to investigate this question, since the addition of serum for either factor would suppress the motility of any form containing that antigen, thus rendering impossible the recovery of a culture which contained both factors.

Variation induced by agglutinating serum has been used to study the inter-transformability of the cultures. As mentioned above, LC54A could be transformed to a culture apparently identical with LC54B. This change was accomplished only with some difficulty. It was even more difficult to change the induced form back to a culture resembling LC54A, from which it was derived. This shifting of a culture from one form to another and then reversing the process bears not the least resemblance to the normal phase variation of Andrewes (1922) in which each phase naturally gives rise to the other. Although the z_{30} form could be transformed to z_{31} , as yet it has not been possible to change any of several z_{31} substrains to z_{30} . The only change in that direction was the one mentioned in which the z_{31} form was obtained from a z_{30} variant and then reversed. This lack of ready transformation of the variants under the influence of serum indicates that such a change would occur only rarely in nature. It was

possible to change N153 and *S. hormaechei* 4 to cultures resembling *S. hormaechei* 1 by cultivating them in N153 serum absorbed by *S. hormaechei* 1. If N153 or *S. hormaechei* 4 were cultivated in N153 serum absorbed by boiled organisms of the homologous strain, they could be transformed into cultures like LC54B. These observations indicate that *S. hormaechei* 1 and *S. hormaechei* 4, both of which presumably came from the same culture, are loss variants of a complex parent, just as are LC54A and LC54B. It is also possible to obtain from *S. ballerup* (XXIX [Vi]: z_{14}) an induced variant which closely resembles LC54B. From this it might be argued that *S. ballerup* as well as the cultures under discussion were all derived from a common source. These observations on induced variation confirm the investigation of Monteverde, who further found it possible to obtain a culture which resembled *S. ballerup* from LC54. In the present investigation, insufficient work has been done on induced variation to draw any definite conclusions regarding the genetic relationship of *S. ballerup* to the organisms under discussion.

In addition to the cultures described here, two other cultures apparently identical with *S. hormaechei* 4, N153, and LC54A were recognized. One of these was isolated by Dr. James Watt from the stool of a child affected with diarrhea. The other was received from Dr. Robert Graham and was isolated from the intestine of a hog that had colitis. It is evident that such forms are not mere curiosities but that they occur naturally in infections of man and animals. If one encountered such cultures and was unaware of their relationship to LC54 and of the variation which occurred in that culture, the natural conclusion would be that a new *Salmonella* type was involved. Also, if one were dealing with LC54A and LC54B and knew nothing of their origin, it would be logical to assume that they were two distinct types with identical somatic antigens but with unrelated flagellar components. This gives further support to the theory of White (1926) that *Salmonella* types arise by loss variation. Edwards and Bruner (1939, 1942) and Bruner and Edwards (1941) studied loss variation in *Salmonella paratyphi* A, *Salmonella abortus-equi*, and *Salmonella salinatis*. In the case of the first two types it was possible to recover suppressed phases by induced variation, indicating that the monophasic organisms which we know today were derived from diphasic forms. In *S. salinatis*, which was naturally diphasic, it was possible to remove the same major component from both phases by induced variation and to create a simpler form which is indistinguishable from *Salmonella sandiego*. The present work is unlike that cited above since apparently it has no connection with phase variation as that term is ordinarily employed. In this case an organism which appears to be monophasic in the ordinary sense gives rise to two distinct and seemingly stable loss variants. The possible relationships of some of the presently recognized monophasic *Salmonella* types should be considered in the light of these results.

SUMMARY

Salmonella hormaechei (XXIX [Vi]: z_{30} , z_{31}) of Monteverde was found to dissociate two loss variants represented by the formulas XXIX [Vi]: z_{30} . . . and

XXIX [Vi]:z₃₁ The two variants had no major H antigens in common, but in combination they possessed all the antigens of the parent culture. The variants were apparently stable types which arose through a hitherto undescribed form of variation. The possible bearing of this variation on the origin of *Salmonella* types was briefly discussed.

ACKNOWLEDGMENT

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GLASS BACTERIOLOGICAL FILTERS ARRANGED FOR POSITIVE PRESSURE¹

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In spite of the advantages of positive pressure filtration in both chemical and bacteriological work, negative pressure seems to be almost universally used in the laboratory. Positive pressure not only reduces or eliminates evaporation of the filtrate, but greatly facilitates the interchange of receivers—particularly important in bacteriological filtrations which must be handled with sterile technique.

In this laboratory various positive pressure setups have been made during the last few years in which Jena 53 filters were used with rubber stoppers. These are not entirely satisfactory because the compressed air leaks out. It was not until Corning UF filters became available recently that it was feasible to construct apparatus with ground joints. The drawing, figure 1a, illustrates an apparatus which I have asked the Corning Glass Works to make. In this figure *B* is the main body of the filter, *A* is a shield to protect the receiver from dust, and *C* is a "pressure head" carrying a stopcock. *C'* is an alternate "pressure head" with a built-in mercury manometer. The stopcock on *C* (*C'*) allows the retention of pressure after the apparatus is detached from the source of compressed air. The shield *A* may be made longer if it is desired to protect receivers for long periods of time. (Similar shields without ground joints can be fitted to filter stems with rolled cotton gauze "stoppers.") Figure 1b is a photograph of an apparatus I have made from a Corning 15 UF filter. The latter construction differs from the drawing in that the small filter is sealed inside the shield and the "pressure head" is provided with an extra ground joint connection to facilitate filling the manometer with mercury. The "pressure head" could be made in other ways—for instance, with a small metal gauge in place of the mercury manometer. A pressure indicator is not essential but is very helpful in controlling the filtering. The sealed-in manometer gives the advantage that the combination can be moved about readily; for example, it is easy to conduct filtrations in an icebox. Without a manometer the rate of filtration may or may not be an index of the pressure.

An ordinary rubber pressure bulb is satisfactory for producing pressures up to at least 450 mm Hg, and the apparatus will hold this pressure for days. To enable it to do this, strong rubber bands or springs are needed, and the joints and stopcocks must be lubricated with a good grease such as "lubri Seal" or, for low or high temperatures, "high-vacuum silicone."

The 15 UF filters allow the passage of distilled water at about 0.5 to 1 ml per minute at 250 mm Hg pressure, a rate similar to that obtainable in the centrifuge at 1,500 rpm (Bush, Goth, and Dickison, 1945).

We have found the positive pressure arrangement very convenient for the

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preparation of sterile solutions of antibiotics preliminary to dilution assays. During the study of the purification of such substances it is usually desirable to assay many fractions, and this requires many filtrations. A Corning UF or a Jena 5/3 filter can be used to make one such filtration after another without resterilizing; in such a series of filtrations the ease with which sterile receivers can be changed under the pressure filter is a great aid in maintaining sterility. It may be desirable to wash the filter between filtrations; if the "holdup" is

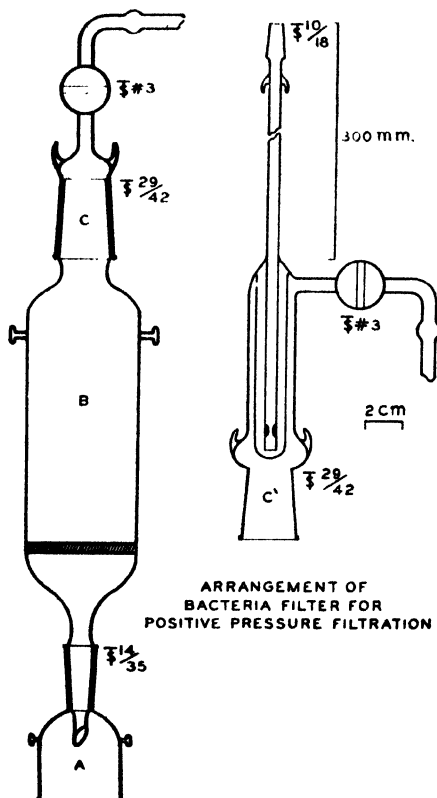


FIG. 1a

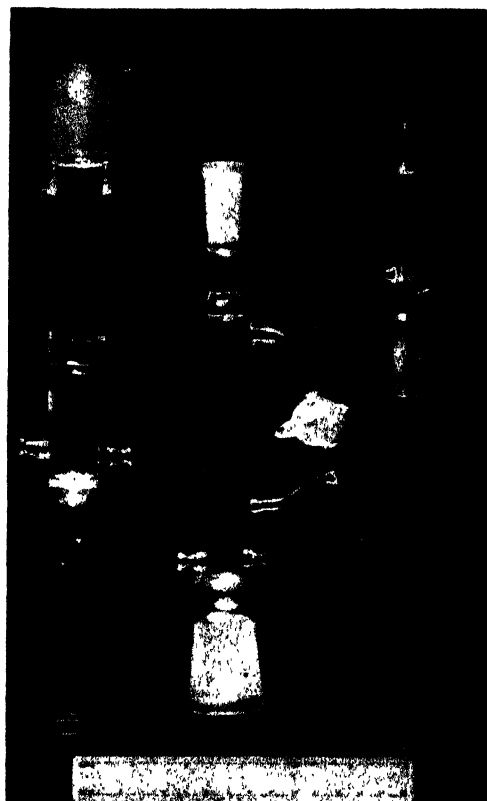


FIG. 1b

large enough to interfere with the accuracy of dilutions it is convenient to use graduated test tubes for receivers and to make final precise dilutions in these.

In our experience the glass filters do not let bacteria through during prolonged filtrations if the solutions being filtered do not provide good growing conditions. If such a solution provides a good medium for bacterial growth, the pressure filter is readily set up in the icebox. If the filter is washed with distilled water immediately after use, ordinary air-borne contaminants apparently cannot "grow through" the filter. A week later sterile filtrates have been obtained without resterilizing.

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EFFECT OF STAPHYLOCOCCUS AUREUS EXTRACTS ON VARIOUS BACTERIA

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For some time past, reports dealing with the effect of protein-free alcoholic extracts of various animal tissues on the growth of certain bacteria *in vitro* and *in vivo* have come from these laboratories (Nutini and Kreke, 1942; Nutini, Kreke, and Schroeder, 1945; Nutini and Lynch, 1945). The effectiveness of such extracts suggested further research using extracts of specific bacteria instead of the animal tissues. These bacterial extracts, by the nature of their preparation, differ from the bacterial filtrates investigated by other workers. Observations on the manner in which bacterial filtrates affect the growth of microorganisms have been made since the time of Pasteur. Within relatively recent years research concerned with bacteriostatic agents, initiated by Dubos (1939), has been carried on by such workers as Hettche and Weber (1939), Sarnowicz (1939), Waksman and Woodruff (1940), and Hotchkiss and Dubos (1941). The influence of living cells of one bacterial strain on the life processes of another grown in its presence has also been studied extensively by a number of workers, among whom may be mentioned McLeod and Govenlock (1921), Dujardin-Beaumetz (1932), Duliscouet (1935), and Waksman and Woodruff (1940; 1942). The literature has been briefly reviewed by Stokes and Woodward (1942) and exhaustively studied by Waksman (1945).

It was the purpose of the present investigation to test the effect on bacterial growth *in vitro* of (a) protein-free alcoholic extracts of *Staphylococcus aureus* cells alone, (b) protein-free alcoholic extracts of medium obtained from ultraviolet-irradiated and nonirradiated cultures of *Staphylococcus aureus*, and (c) filtered, sterilized, but unextracted medium from ultraviolet-irradiated and non-irradiated cultures of *Staphylococcus aureus*.

METHODS

Source of Staphylococcus aureus. The culture of *Staphylococcus aureus* was one isolated from an infected tonsil (Pathological Laboratory, Good Samaritan Hospital, Cincinnati, Ohio). It was the same strain as that used for the test organism in experiments employing animal tissue extracts in these laboratories (Nutini and Lynch, 1945).

Cell extract. The cell extract was prepared as follows: Large Roux flasks containing 250 ml of nutrient agar were inoculated aseptically with 3 ml of a 24-hour-old broth culture of *Staphylococcus aureus*, plugged, and incubated for 48 hours at 37 C. At the end of that time the bacterial cells were washed from the

agar surface with 20 ml normal saline. The washings were subjected to the procedures previously described for animal tissues (Nutini and Kreke, 1942).

Extracts of medium from nonirradiated cultures. Erlenmeyer flasks (250 ml) containing 100 ml of sterile nutrient broth were inoculated with 0.1 ml of a 24-hour-old broth culture of *Staphylococcus aureus* and incubated at 37 C for 48 hours. The bacterial cells were then removed by centrifugation. An extract of the supernatant broth was prepared exactly as for the cells with the exception of the omission of the freezing and thawing, which was considered unnecessary in the absence of cellular material. In order to determine whether active substances were adsorbed by the filters, some extracts were sterilized by using Seitz and others by using Berkefeld filters.

Extracts of medium from irradiated cultures. Large quantities of 48-hour-old broth cultures of *Staphylococcus aureus* were introduced into special large glass (Corning 9741, grade 3) tubes and exposed to nonfiltered ultraviolet radiation from a Burdick A.C. quartz mercury arc at a distance of 45 cm for 24- and 48-hour periods. The tubes were rotated from time to time. After centrifugation the supernatant broth was divided into two parts and an alcoholic extract prepared from one part.

Filtrates of irradiated cultures. The second portion of the irradiated broth was sterilized by Seitz filtration in order to compare the effectiveness of the irradiated, protein-free alcoholic extracts of medium with that of untreated, irradiated, sterilized filtrates.

Filtrates of nonirradiated cultures. The nonirradiated broth medium of 48-hour-old cultures was centrifuged and the supernatant broth sterilized.

Measurement of activity. Bacterial growth in terms of increase or decrease in colony numbers was estimated by the pour plate method. To 15 ml of warm, sterile nutrient agar, volumes of the extract or filtrate to be tested were added aseptically to give concentrations of 0.1, 0.5, 1.0, and 5.0 per cent. The extract or filtrate was thoroughly mixed through the agar by rotating the tube. It was then poured into petri plates containing 0.1 ml of a 1:10,000 dilution of a 24-hour broth culture of the test organisms, *Staphylococcus aureus*, *Escherichia coli*, strain 4265, *Aerobacter aerogenes*, strain 211, and *Shigella dysenteriae*, strain 9665. (The last three organisms were from the American Type Culture Collection, but the strain of *Staphylococcus aureus* was the same as that used in making the extracts and filtrates.) The plates were incubated for 48 hours at 37 C, and growth was determined by making colony counts on a Wolfhuegel plate counter. Control organisms were cultured in plates containing agar only, or agar and a sterile nutrient broth in the same percentage of concentration as was used for the experimental plates.

Colony counts for the control plates were taken as 100 per cent, and the difference between these counts and those obtained on the experimentals was calculated. Results with the pour plate method are subject to about 25 per cent experimental error, so that only extracts producing stimulatory or inhibitory effects in terms of colony numbers lying well beyond this range were considered significant. Triplicate experiments were conducted. In several experiments Hopkins

tubes were also used as checks, but the cell volume was too small for accurate reading.

Investigations were also undertaken to determine whether changes had occurred in the biochemistry of the test organisms. These experiments consisted of the fermentation of lactose, sucrose, and glucose; coagulation of litmus milk; liquefaction of gelatin; indole formation; and nitrate reduction. Only *Staphylococcus aureus* cell extract and the Seitz-filtered broth extract were used in these experiments.

TABLE 1

Effect of extracts of Staphylococcus aureus cells and of irradiated and nonirradiated broth media on the growth of organisms

TYPE OF EXTRACT USED	PER CENT	E. COLI	A. AEROGENES	S. AUREUS	S. DYSENTERIAE
Cell extract	0.1	No effect	Inhibition	Complete inh.	Complete inh.
	0.5	Slight inh.	Complete inh.	Complete inh.	Complete inh.
	1.0	Inhibition	Inhibition	Complete inh.	Stimulation
	5.0	No effect	Inhibition	Stimulation	Stimulation
Broth extract, Seitz filter	0.1	No effect	Inhibition	No effect†	No effect
	0.5	No effect*	Slight inh.	No effect†	Inhibition
	1.0	No effect*	Slight inh.	No effect†	Inhibition
	5.0	Stimulation	Slight inh.	No effect	Inhibition
Broth extract, Berkefeld filter	0.1	Slight stim.	No effect	No effect	No effect
	0.5	No effect	No effect	No effect	No effect
	1.0	No effect	No effect	No effect	No effect
	5.0	No effect	No effect	No effect	Slight inh.
Extract of 24-hr. irradiated broth, Seitz filter	0.1	Stimulation	Inhibition	Inhibition	No effect
	0.5	Stimulation	Slight inh.	Inhibition	Stimulation
	1.0	Stimulation	Slight inh.	Inhibition	Stimulation
	5.0	Slight stim.	Inhibition	Inhibition	Stimulation

Control colonies, 100 per cent; slight stimulation, 125 to 175 per cent; slight inhibition, 75 to 25 per cent; inhibition, 25 to 0 per cent; stimulation, 175 to 500 per cent; no effect, 75 to 125 per cent.

* Prevented coagulation of milk.

† Coagulated milk.

RESULTS

The effects of several concentrations of *Staphylococcus aureus* extracts and filtrates on the growth of 4 test bacteria are given in tables 1 and 2. A survey of the data shows that the growth response varied with the species of the test organism, the material tested, and its concentration, as well as with the irradiation of the *Staphylococcus aureus* cultures from which the test materials were prepared. The mode of filtration, whether by Seitz or Berkefeld, produced no appreciable difference in the response of the organisms to the broth extracts and filtrates.

Cell extract. Extracts of *Staphylococcus aureus* cells had a predominately inhibitory effect on the growth of the 4 test organisms. The exceptions were in the stimulating action of higher concentrations of the cell extracts on *Staphylococcus aureus* and *Shigella dysenteriae*.

Broth extracts. The broth extracts, with the single exception of growth stimulation of *Escherichia coli* by a 5 per cent concentration, had no effect or inhibited growth of the test organisms. It is apparent from table 1 that extracts produced from broth media of 24-hour irradiated *Staphylococcus aureus* cultures differed in their action from nonirradiated preparations.

Filtrates. The action of the simple filtrates, on the other hand, was essentially one of stimulation of growth. Filtrates of 24-hour irradiated cultures of *Staph-*

TABLE 2

Effect of filtrates of irradiated and nonirradiated broth media in which Staphylococcus aureus was cultured

TYPE OF FILTRATE	PER CENT	E. COLI	A. AEROGENES	S. AUREUS	S. DYSENTERIAE
Filtrate of nonirradiated broth	0.1	Stimulation	No effect	No effect	No effect
	0.5	Stimulation	No effect	No effect	No effect
	1.0	Stimulation	Slight inh.	Stimulation	Slight stim.
	5.0	Slight stim.	Slight inh.	Stimulation	Stimulation
Filtrate of 24-hr. irradiated broth	0.1	Slight inh.	Inhibition	No effect	No effect
	0.5	No effect	Inhibition	Slight stim.	No effect
	1.0	No effect	Inhibition	Slight stim.	No effect
	5.0	No effect	Inhibition	Stimulation	Inhibition
Filtrate of 48-hr. irradiated broth	0.1	Stimulation	No effect	No effect	No effect
	0.5	Stimulation	No effect	Slight stim.	Slight stim.
	1.0	Stimulation	No effect	Stimulation	No effect
	5.0	Slight inh.	No effect	No effect	No effect

See footnote to table 1 for definition of terms of response.

Staphylococcus aureus differed somewhat in their effect on the growth of the 4 test organisms from nonirradiated filtrates. After 48-hour irradiation of cultures of *Staphylococcus aureus* the action of broth filtrates on the growth of organisms was essentially the same as that of filtrates of nonirradiated cultures.

Biochemical changes. Observations on biochemical tests showed that the presence of the cell or broth extracts did not interfere with the fermentation of sugars, indole formation, nitrate reduction, or the liquefaction of gelatin. Broth extract, as shown in table 1, prevented coagulation of milk by *Escherichia coli*. The coagulating system was also affected in *Staphylococcus aureus*, which, like other strains obtained from similar sources, does not coagulate milk. Coagulation was observed in the presence of the broth extract, as indicated in table 1.

In vitro and *in vivo* investigations of extracts of *Staphylococcus aureus*, as well as those from other organisms, are under way in these laboratories.

DISCUSSION

As Waksman (1944) points out, such bacterial preparations as those described here may affect one or more of the metabolic functions of the bacterial cell which is submitted to investigation. Among the probable mechanisms involved are interference with cell division, modification of the respiratory and other enzymatic systems, and changes in the surface tension of the cell and in the osmotic pressure, with accompanying changes in the utilization of nutrient materials and growth-regulatory substances.

The results, though inconclusive, add evidence to Waksman's (1944) observation that antibiotic substances are selective in their action on bacteria. This investigation also confirms the report of Loofbourow and Morgan (1940) that different concentrations of the same cellular extract can produce opposite effects on bacterial growth.

SUMMARY

Several protein-free alcoholic extracts of a virulent strain of *Staphylococcus aureus* cells and of filterable bacterial products in the media in which they were grown were prepared. The effects of these and simple filtrates on the growth of *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, and *Aerobacter aerogenes* were investigated.

Extracts of both cells and the media in which they were grown, while showing some slight differences, were predominantly inhibitory in their growth effects on the test organisms. Cell-free filtrates of media in which *Staphylococcus aureus* was grown were, in general, stimulatory in their action on the growth of the test organisms.

Ultraviolet irradiation of *Staphylococcus aureus* cultures slightly modified the action of both the broth extract and the filtrates.

There was a reversal of the growth response of the test organisms with higher concentrations of the cell extract (2 instances) and filtrates of the 48-hour ultraviolet irradiated cultures of *Staphylococcus aureus* (1 instance).

Biochemical tests indicate that in some cases the alcoholic broth extract may interfere with the coagulase reactions of the bacteria in culture.

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ATYPICAL ACID-FAST MICROORGANISMS

II. DESOXYRIBONUCLEIC ACID CONTENT

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Since the discovery of nucleic acid by Miescher, the studies of many investigators have contributed to our knowledge of the chemical structure of these substances and their distribution in the cells of animals, plants, and bacteria. The two principal groups of nucleic acid are desoxyribonucleic acid and ribonucleic acid, the former occurring in the nucleus like particles of fungi and bacteria and in the nuclei of the cells of higher plants and animals, and the latter in the cytoplasm of the cells of plants, animals, and bacteria. The investigations of Johnson and Brown (1922) and Brown and Johnson (1923) of the nucleic acid of tubercle bacilli have shown it to be of the desoxyribose type. On the other hand, Coghill (1931) found the nucleic acid of the timothy bacillus to be of the ribose type and suggested that the determination of the nucleic acid content might furnish a basis for the chemical classification of bacteria. In view of this difference in the type of nucleic acid of these bacteria it was felt that a study of the nucleic acid of atypical acid-fast bacilli isolated from the sputa and gastric contents of patients with proved or suspected tuberculosis (Loesch and Petrik, 1939) might be of value in correlating these microorganisms with tubercle bacilli and thus aid in evaluating the significance of their presence in sputa and other excreta. A preliminary qualitative study (Petrik, 1944) revealed that six strains of atypical acid-fast bacilli contained the desoxyribose type of nucleic acid when tested by the Dische (1930) reaction for desoxyribose, and that it was present in low concentration or absent entirely in four other strains. The present report deals with the quantitative estimation of nucleic acid in a number of strains of *Mycobacteria*.

MATERIALS AND METHODS

The *Mycobacteria* used in this study were three strains of atypical acid-fast bacilli, Ho, He (white), and Mar (orange), two strains of *Mycobacterium phlei*, one strain of *Mycobacterium thamnophaeos*,¹ and one strain of *Mycobacterium tuberculosis* var. *hominis*, Co, isolated from the sputum of a patient with pulmonary tuberculosis. This organism has a low virulence for guinea pigs. All the microorganisms were grown on the Long synthetic medium described previously (Petrik, 1944), except *M. phlei*, which was also cultivated on Henley's synthetic medium (Henley, 1929). The cultures were incubated at 37 C, ex-

¹ The strains of *M. phlei* 8a and *M. thamnophaeos* 75 of the Cornell Veterinary College Collection were obtained through the courtesy of Dr. W. D. Bellamy. The Parke Davis and Co. kindly supplied the strain of *M. phlei* 02145 used to grow the organisms for Dr. R. D. Coghill's investigation in 1931.

cept *M. thamnopheos*, which was grown at room temperature, for 4 to 8 weeks, harvested by filtration, washed once with distilled water, and then extracted with alcohol-ether, ether, and finally with chloroform-ether over a period of about 1 month.

Preparation of nucleic acid. In general, the methods used for preparing nucleoproteins employ treatment with distilled water, sodium chloride solutions, or dilute alkali solutions, followed by the precipitation of the nucleoprotein by the addition of alcohol or acid to the clear solution of the nucleoprotein. The isolation of pure nucleic acid in relatively intact form is readily accomplished from some nucleoproteins because of the nature of the bond between the nucleic acid and protein. Mirsky (1943), in discussing the cellular nucleoproteins, states that "the type of bond between protein and nucleic acid is different in ribose nucleoprotein from what it is in desoxyribose nucleoprotein. In the latter the linkage appears to be primarily of a saltlike nature. The bond between nucleic acid and protein in naturally occurring ribose nucleoprotein is nonpolar. It is readily broken by some of the procedures that have frequently been used to prepare these nucleoproteins. Just what the bond is remains obscure, but it certainly is different from the saltlike linkage formed when the separated components of a ribose nucleoprotein are brought together." Bang (Mirsky, 1943) found that nucleohistone of thymus prepared by extraction with water could be split into nucleic acid and histone by saturation with sodium chloride. On the other hand, Taylor *et al.* (1942) found it necessary to use more stringent methods to break the bond between nucleic acid and protein in rabbit papilloma virus protein. The protein residue from cold alkaline hydrolysis was taken up in 5 per cent sodium hydroxide solution and heated on a boiling water bath for 30 minutes and neutralized. A desoxyribose type of nucleic acid was isolated from the solution, and no evidence was seen of the presence of ribonucleic acid. Johnson and Brown (1922) and Coghill (1931) used dilute alkali solutions during the preparation of nucleic acid from the tubercle bacillus and *M. phlei*. Heidelberg and Scherp (1939) found that the nucleic acid (ribose type) present in a fraction obtained from a scarlatinal strain (C203) of hemolytic *Streptococcus*, group A, is bound more firmly than in dissociable salt linkage. Sevag and Smolens (1941) found the linkage in natural nucleoprotein of *Streptococcus pyogenes* to be nonpolar rather than polar, thus requiring treatment with alkali, acid, or certain organic solvents to split or weaken the linkage of the natural nucleoprotein. Brues, Tracy, and Cohn (1944) warn against the use of the method of alkaline hydrolysis described by Levene and Bass (1931), claiming that this method cannot be used to obtain desoxyribose nucleic acid without at the same time hydrolyzing any ribose nucleic acid that may be present, owing to the instability of the latter in alkaline solution.

Since the investigations of a number of workers indicated that both types of nucleic acid may be present in bacteria, a number of methods were used in the attempt to determine the nucleic acid content of the *Mycobacteria* used in this study: (1) The method described by Coghill (1931) for the timothy bacillus. (2) Levene's (Levene and Bass, 1931) method for desoxyribonucleic acid, and

purification by the ammonia acetic acid method. (3) Extraction of the nucleoprotein with molar sodium chloride as suggested by Mirsky and Pollister (1942), followed by the precipitation of the nucleoprotein with alcohol. The methods used to split the bond between nucleic acid and protein were (a) treatment with 5 per cent sodium hydroxide for 2 hours at 0 C as suggested by Johnson and Harkins (1929), (b) treatment with 5 per cent sodium hydroxide for 1 hour at 0 C, and (c) the method of Cohen and Stanley (1942), which avoids the use of alkali, the solution of nucleoprotein in 0.1 N sodium chloride at pH 5 to 6 being heated for one minute at 100 C. The reactions of the hydrolyzed nucleoprotein solutions were adjusted to pH 6.8 and the nucleic acid was precipitated by the Jorpes barium acetate method and purified by the Levene-Jorpes acetic acid method

TABLE 1
Comparison of methods for isolating nucleic acid

STRAIN	METHOD	YIELD	DI-PHENYL-AMINE RE-AGENT	NITROGEN	PHOSPHORUS	NITROGEN: PHOSPHORUS ratio
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
<i>M. phlei</i>	Coghill	0.630	102	14.42	8.60	1.67
<i>M. phlei</i>	Levene	0.680	105	13.44	8.40	1.60
<i>M. phlei</i>	3a	0.660	102	13.96	8.27	1.68
<i>M. phlei</i>	3b	0.720	100	14.84	8.60	1.72
<i>M. phlei</i>	3c	Bond not split				
<i>M. phlei</i>	Bang	Bond not split				
Thymus nucleic acid*			100	14.28	8.2	1.74
Yeast nucleic acid (Pfanstiehl)			0	14.35	8.38	1.71

* The sample of thymus nucleic acid for this investigation was kindly supplied by the Difco Laboratories, Inc.

(Levene and Bass, 1931). (4) The method suggested by Bang (Mirsky, 1943), i.e., saturation with sodium chloride.

Determination of nucleic acid. In order to determine which of the foregoing methods was the most suitable for extracting the nucleic acid from the Mycobacteria, 5-gram portions of dried, defatted *M. phlei* bacilli were ground in a mortar to a fine powder and extracted by each of the methods described above. The purified nucleic acid was tested for pentose with the orcinol reagent and for desoxyribose with Dische's diphenylamine reagent. Nitrogen was determined by the micro-Kjeldahl method and phosphorus by the method of Fontaine (1942). The results obtained are summarized in table 1. The only type of nucleic acid isolated by each one of the four methods was the desoxyribose type, as shown by the blue color (characteristic of desoxyribose) obtained with the diphenylamine reagent. This test is considered highly specific and has been used exclusively by a number of investigators for estimating desoxyribonucleic acid. The tests for pentose were negative with the orcinol reagent. Tests for thymine could not

be done because some of the materials needed for the reagents were not available. The values for the nitrogen-phosphorus ratio are consistent with those required by theory for the tetranucleotide structure of nucleic acid. No free nucleic acid could be precipitated when the nucleoprotein solutions were treated by method

TABLE 2

Part 1. Effect of the composition of the medium on the nucleic acid content
Part 2. Nucleic acid content of two strains of Mycobacteria

STRAIN	METHOD	YIELD	DI-PHENYL-AMINE RE-AGENT	ORCINOL RE-AGENT	NITROGEN	PHOSPHORUS	NITROGEN: PHOSPHORUS
Part 1							
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>ratio</i>
<i>M. phlei</i> *	3a	0.645	102	0	14.31	8.43	1.69
<i>M. phlei</i> †	Levene	0.580	106	0	14.44	8.52	1.68
<i>M. phlei</i> †	3a	0.690	90	13	14.32	8.36	1.71
Thymus nucleic acid			100	0	14.28	8.20	1.74
Yeast nucleic acid (Pfanstiehl)			0	100	14.35	8.38	1.71
Part 2							
<i>M. tuberculosis hominis</i> , Co.	3a	0.560	65	45	13.86	8.01	1.73
Ho.	3a	0.405	82	24	13.79	8.67	1.59

* Cultivated on a Long's synthetic medium.

† Cultivated on Henley's synthetic medium.

TABLE 3

Desoxyribonucleic acid content of five strains of Mycobacteria

STRAIN	YIELD	DIPHENYL-AMINE REAGENT	NITROGEN	PHOSPHORUS	NITROGEN: PHOSPHORUS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>ratio</i>
<i>M. thamnopheos</i>	1.250	101	14.14	8.30	1.70
He.	0.230	100	14.07	8.32	1.69
Ho.	0.310	105	14.80	8.70	1.70
Mar.	0.410	101	11.90	7.30	1.63
<i>M. phlei</i>	0.680	105	13.47	8.40	1.60
Thymus nucleic acid		100	14.28	8.20	1.74
Yeast nucleic acid (Pfanstiehl)		0	14.35	8.38	1.71

3c or by that of Bang, probably because the bond between the nucleic acid and protein was not split or weakened sufficiently to allow the nucleoprotein to dissociate into its components. Since these results did not agree with Coghill's finding of the so-called plant or ribose type of nucleic acid in the timothy bacillus, a strain of the culture used to grow the organisms for his investigation was obtained from Parke Davis and Company to determine whether it differed from the strain used in this study. This organism was cultivated on the Long syn-

thetic medium and also on Henley's synthetic medium (the latter contains glucose in addition to glycerol) to determine whether the composition of the medium would have any effect on the nucleic acid content of the bacilli. The methods used to extract the nucleic acid were Levene's and 3a, and the results of this study are summarized in the first part of table 2. The desoxyribonucleic acid content of this strain appears to be similar to that of the Cornell strain. The presence of a small amount of pentose sugar in the material extracted by method 3a indicates that a small amount of ribonucleic acid may also be present.

Determination of desoxyribonucleic acid. Five-gram portions of *M. thamnophaeos*, *M. phlei* (Cornell strain), and atypical strains He, Ho, and Mar were extracted by Levene's method, and the nucleic acid was purified by the ammonia acetic acid method. The results of this study are summarized in table 3. The desoxyribose type of nucleic acid was found in each of the strains examined, as is shown by the reaction with the diphenylamine reagent.

Estimation of the nucleic acid content of the atypical strain Ho, and M. tuberculosis var. hominis strain Co. Five-gram portions were extracted by method 3a, and the results are summarized in the second part of table 2. This method appears to be satisfactory for isolating both types of nucleic acid.

DISCUSSION

The results of this study and those of other investigators indicate that the desoxyribose type of nucleic acid is probably present in all strains of *Mycobacteria*, and that strains of known virulence for animals may have a greater content than the so-called avirulent strains. The positive test for pentose with the orcinol reagent indicates that the ribose type of nucleic acid was also present in the material obtained from three of the strains investigated. The difference in the results of this investigation from those of Coghill may be due to (1) a variation in the strain of *M. phlei*, (2) the chemical composition of the culture medium on which the organism was grown, or (3) the age of the culture at the time of harvesting. Creighton, Chang, and Anderson (1944) have shown that when the H37 strain *M. tuberculosis* is cultivated on a modified Long synthetic medium in which glucose replaces glycerol, the fat contained no glycerol, but a carbohydrate which could not be identified. Recent investigations (Mirsky, 1943) have shown that a greater concentration of the ribose type of nucleic acid is found in the cytoplasm of rapidly dividing cells of tissues and yeast. That the desoxyribose type of nucleic acid is found in these strains of *Mycobacteria* is not surprising since evidence is accumulating (Allen, 1941) to support the view that this substance may be found in all nuclei regardless of origin, whereas the ribonucleic type occurs mainly in the cytoplasm. Recently Avery, MacLeod, and McCarthy (1944) reported a highly polymerized, viscous form of desoxyribonucleic acid isolated from encapsulated type III pneumococci which was capable of inducing the transformation of noncapsulated type II pneumococci into fully encapsulated type III organisms. Whether or not the desoxyribonucleic acid of the *Mycobacteria* can induce transformations in this group of bacteria remains to be demonstrated. One difficulty that may be encountered is that the more drastic methods re-

quired to split the bond between nucleic acid and protein in the nucleoproteins isolated from *Mycobacteria* may destroy or alter the transforming principle. The possibility of desoxyribonucleic acid or of the intact nucleoprotein influencing *Mycobacteria* in a way similar to that reported for pneumococci is being studied. The influence of the age of a culture and the rapidity of growth on the nucleic acid content of the *Mycobacteria* is under investigation.

SUMMARY

The desoxyribonucleic acid content of a number of strains of *Mycobacteria* was determined. It is probable that this type of nucleic acid will be found in all strains of this microorganism.

The reaction for pentose obtained with the orcinol reagent indicated that ribonucleic acid was also present in three of these strains.

The nucleic acid content of the two strains of *Mycobacterium phlei* studied was found to consist largely of the desoxyribose type, which differs from Coghill's finding of the ribose type.

The values for the nitrogen-phosphorus ratios are consistent with that required by theory for the tetranucleotide structure of nucleic acid.

The bond between nucleic acid and protein in mycobacterial nucleoprotein apparently is nonpolar since it was necessary to treat the nucleoprotein with alkali before the nucleic acid could be precipitated free of protein.

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THE INFLUENCE OF HEAVY WATER ON THE GROWTH, MORPHOLOGY, AND FERMENTATION REACTIONS OF *EBERTHELLA TYPHOSA*¹

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Since the discovery of heavy water, many experiments have been made to determine its influence upon living organisms. Although some of these investigations have shown little effect (Melot, 1934; Rea and Yuster, 1934), others (Lewis, 1933; Harvey, 1934; Ussing, 1935) reported a depressing action on the growth of plants and animals. The pioneer experiment in this field was carried out by Lewis (1933). Tobacco seed (*Nicotiana tabacum* var. *purpurea*) would not germinate in 100 per cent heavy water but did germinate very slowly in a 50 per cent concentration. Pratt and Curry (1937) found that wheat seedlings and the lower parts of buds of *Kalanchoe daigremontiana* grew only 0.025 as rapidly in 99 per cent heavy water as in normal water. The osmotic effects of heavy water on the leaf cells of *Nitella clavata* was studied by Brooks (1937), who found that the cells shrank in heavy water and expanded in normal water. It was concluded that heavy water was hypertonic to the cells.

Fox, Cupp, and McEwen (1936) measured the growth of diatoms in 1 per cent heavy water and in filtered sea water. They reported a lag of 16 per cent in the growth rate of *Nilzschia bilabata* over a period of 12 days in heavy water. Freshly collected *Spirogyra* placed in 0.06 per cent heavy water by Barnes (1933) was characterized by lack of movement, much less cell disjunction, and greater longevity than the controls in distilled water. A study of the influence of heavy water upon the rate of photosynthesis was made by Craig and Trelease (1937) upon *Chlorella vulgaris* suspended in a carbonate-bicarbonate buffer. Using the evolution of oxygen as a measure of photosynthesis, they found a decrease in the rate of 0.41 in 99.9 per cent heavy water. Using the same organism, Pratt (1938) found the decrease in growth to be in inverse linear proportion to the concentration of deuterium oxide up to 75 per cent, at which concentration practically no growth occurred.

Taylor, Swingle, Eyring, and Frost (1933) showed that 92 per cent of heavy water influenced the life processes of tadpoles, *Rana clamitans*; fish, *Lebistes reticulatus*; flatworms, *Planaria maculata*; and the protozoan, *Paramecium caudatum*. The tadpole died within an hour; the fish within 2 hours; the flatworm within 3 hours; but the *Paramecium* lived 48 hours. When the concentration was decreased to 30 per cent, no effect could be detected on the tadpoles, fish, or flatworms over a period of 48 hours. When the white mouse was subjected (Barbour, 1935) to a 99 per cent solution of heavy water, the metabolism was slowed

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down to about 50 per cent of normal. The rate of metabolism was also slowed in *Paramecium*, as shown by Barnes and Gaw (1935) when the organism was placed in a 30 per cent solution of heavy water.

According to Barnes (1935), the life span of many primitive cells was increased when they were subjected to dilute solutions of heavy water, and the general biological processes were slowed down in water containing 30 to 100 per cent deuterium oxide. Lockemann and Leunig (1934) studied the effect of heavy water upon *Escherichia coli* and *Pseudomonas aeruginosa*. Concentrations of less than 0.54 per cent were found to favor growth. The oxygen consumption of luminous bacteria (*Vibrio phosphorescens*) in heavy water was studied by Harvey and Taylor (1934). It was found that the rate of respiration of these organisms decreased in proportion to the concentration. The rate was slowed down 12 per cent at a concentration of 36 per cent, and 60 per cent when 63 per cent heavy water was used. Weiser (1937) studied the influence of heavy water on the growth and morphology of 8 strains of lactobacilli. The strains were grown in whey broth containing 0.13 per cent, 0.7 per cent, and 5 per cent. Examination failed to show any differences in growth as compared to controls. At the end of 48 hours, however, 3 strains had become gram-negative and markedly granulated in the 5 per cent solution.

Alcoholic fermentation in heavy water *d*-glucose broth has been reported to be 8 to 9 times slower than when normal water was used in the broth (Pacsu, 1934).

MATERIALS AND METHODS

The heavy water was obtained from the Ohio Chemical and Manufacturing Company, Cleveland, Ohio. It contained 8 per cent of deuterium oxide as tested by the company, using the micro specific gravity method. It was received in sealed glass containers in 100-ml amounts. The water remained in the tubes until ready for use, when it was transferred to glass-stoppered bottles. The organisms, *Eberthella typhosa*, were obtained from the School of Medicine of the University of Oklahoma. Pyrex culture tubes were used. Wassermann size tubes were used in preliminary studies and regular size tubes for final studies. The culture tubes were washed in hot soapy water and rinsed three times in tap water, twice in distilled water, and finally in triple-distilled water. Slides were given similar treatment. The dye solutions were autoclaved immediately after making up. Each time a series of slides was made, a blank smear was made and examined for the presence of microorganisms. Standard Difco media, nutrient agar, *d*-glucose agar, and *d*-glucose broth, were used. It was made up, autoclaved, and allowed to stand at least 24 hours before inoculation and incubation at 37.5 C.

The initial morphological observations were made on cells from nutrient agar slants and *d*-glucose broth made with normal and 8 per cent heavy water. No observable changes were seen in cells from nutrient agar made with either normal or heavy water, but changes were observed in cells from heavy water *d*-glucose agar. The last studies were made on cells from *d*-glucose agar or broth made up with heavy and normal water. The same concentrations of heavy water were

used as in the growth studies. In the early work, nigrosin smear slides were made every hour and later changed to periods of 4, 8, 12, and 24 hours. Hanging drop observations were made upon cells from all concentrations of heavy water *d*-glucose broth and controls. The fermentation reactions of the organism were also tested in the same concentrations of heavy water.

RESULTS AND OBSERVATIONS

Although quantitative tests with *Eberthella typhosa* indicated that growth was more rapid in the presence of certain concentrations of heavy water than in the controls, because of certain technical difficulties this phase of the work is withheld pending further investigation. However, the results obtained parallel those of Lockemann and Leunig (1934) with *Escherichia coli* and *Pseudomonas aeruginosa* as well as those of Kinoshita and Chitani (1935) with *Mycobacterium tuberculosis* var. *hominis* and var. *bovis*.

From the outset gross morphological changes were evident in cells grown on 8 per cent heavy water *d*-glucose agar. Although some variation is to be expected in normal cultures, here it was pronounced. Normal round-ended cells grew into cells with pointed, club-shaped ends and other atypical forms. The shapes of the cells were almost round, oval, bottle-shaped, boat-shaped, large almost rectangular, curved, club, dumbbell, etc. Internally, granulation became conspicuous, whereas in cells from controls it was inconspicuous or absent. Another change was the appearance of small budlike structures on the cells. Atypical cells appeared as early as 4 hours after transfer in heavy water media, whereas such changes were not observed in cells from normal water media. Occasionally, in older cultures aberrant forms were seen in normal media, but with no degree of regularity.

Granulation was very prominent in the aberrant forms. The granules were not only more numerous but appeared to be larger and denser than in cells of the controls. They were observed in any part of the cell though in many cases apparently associated with swollen ends of club-shaped rods. In some instances, it appeared that the granules were associated with bud formation. In hanging drop studies, granules were observed to move to a position adjacent to the wall where a swelling or bulge appeared. This apparently was the initiation of bud formation. Even though nuclei have been reported in "*B. typhi-abdominalis*" by Akimovich (1938), no attempt was made to associate granules with this structure. After further growth, the parent cell lost its densely granular appearance, but the bud then contained granular material. In young cultures, the buds were often thrown off as teardrop-shaped structures, which were highly motile, even exceeding the motility of the parent cell sufficiently to encircle it. Binary fission was observed to be taking place also during the bud formation and development. In cultures 6 to 12 hours old, the cell bearing the larger buds appeared to exhibit the well-known "y" or branching form described by Hort (1917). However, binary fission as the chief method of reproduction held throughout the process of budding, which generally lasted from the third through the twelfth hour from transfer.

Although the growth of *Eberthella typhosa* in heavy water *d*-glucose broth induced morphological disturbances, there was no physiological evidence of change as measured by fermentation reactions. When normal media were inoculated with cells grown in heavy water cultures, the reactions were the same as for cells from the controls. However, some disturbance was evidenced in growth requirements as old (8 to 20 days) heavy water cultures apparently lost certain facilities for growth. It was repeatedly demonstrated that transfers from these old cultures to agar slants would not grow. In order to induce growth on slants, transfers were made to broth and then to agar slants, after which abundant growth occurred.

SUMMARY

A study of the influence of heavy water up to 8 per cent in concentration in nutrient agar, *d*-glucose agar, and *d*-glucose broth was made.

The morphology of the organism was greatly influenced, especially by the higher concentrations in *d*-glucose broth. This was evidenced in cell shape, granulation, and bud formation. Although budding was much in evidence, binary fission remained the chief mode of reproduction.

Fermentation reactions after growth in heavy water were unchanged for the duration of the experiments.

Some growth factor was disturbed since cells from old heavy water cultures would not grow on agar slants but would grow in broth.

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SURVIVAL FOR FOURTEEN YEARS OF AGAR SLANT CULTURES OF ESCHERICHIA COLI-MUTABILE WITHOUT LOSS OF IMPORTANT CHARACTERS

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It is probable that many laboratories have had the experience of having cultures of nonsporeforming bacteria remain viable over a period of years without transplantation. Few reports of such survivals have been recorded in the literature, however. Francis (1943) reported the maintenance of viable *Pasteurella pestis* cultures for 20 years without transfer and with retention of their virulence and their antigenic and biochemical characters. Of 48 beef infusion agar slant cultures, sealed with paraffined corks and stored at 10 C, he found 33 alive at the end of 20 years.

The long-time survivals reported in this paper were of 3 strains of *Escherichia coli*, two of them *mutabile* strains and the third the lactose-fermenting variant derived from one of them. Each strain was inoculated into two beef extract agar slants in October, 1931. The tubes were sealed by pushing the cotton stopper down and inserting a paraffined cork above it. They were stored at room temperature in a black cardboard box. Before being opened for the tests recorded here, each tube had been opened on three separate occasions, tested for viability, and then resealed and stored in the same manner. All 6 cultures were found viable in October, 1945.

The 2 *mutabile* strains were originally isolated from stools and carried the designations 269A and 269B. The variant strain was obtained from the daughter colonies which had developed from 269B on Endo plates and was designated as strain 269Bv. Strains 269A and 269B were known to form acid and gas from lactose broth at 37 C only after incubation for from 3 to 7 days, and to give secondary colonies on lactose agar in about the same length of time. In all other respects both gave the reactions of true *Escherichia coli*, producing indole from tryptophane and giving a positive methyl red test and a negative Voges-Proskauer reaction. Strain 269Bv was identical with the mother strain 269B except that it regularly gave acid and gas in lactose broth in less than 24 hours and did not develop secondary colonies on lactose agar.

At the time of their final opening in October, 1945, all 6 cultures looked very much alike. All the water of condensation had evaporated, and the agar at the bottoms of the slants was slightly retracted from the sides of the tubes. The agar was of a brownish color. The growth appeared as a thin, homogeneous, buttery layer that was moist and glistening and had sharply marked borders. The outer margins of the slant surface, not originally covered with growth, were still visibly bare and the tracks made by the inoculating needle in making previous transplants were still clearly discernible. No secondary colonies or other

evidence of overgrowths was present. When touched with the loop, no stringiness or stickiness could be observed. The material emulsified easily and evenly in broth.

Preparations were made and stained with methylene blue and by Gram's method. In moderately thick films both stains showed the great majority of the organisms to be lightly stained and indefinite in outline. Among these shadowy forms a great many more deeply stained granules of irregular shape appeared. A very few well-formed rods were seen that had the morphology and staining characteristics of young cultures of *E. coli*. These numbered from less than 1 to as many as 10 per microscopic field. All of these forms were decolorized by the acetone-alcohol mixture used in Gram's stain. A moderately thick suspension examined under the dark-field microscope showed myriads of minute, indefinitely shaped particles in active Brownian movement against a background of amorphous material. No whole, normal-appearing organisms could be detected in the dark-field preparation.

Tests for viability and for the important character of variability of the cultures were made by emulsifying two or three loopfuls of the growth from each slant in 10 ml of infusion broth and making transplants immediately from these suspensions to other media. The results of these tests were as follows: 0.1 ml of each of the suspensions from strains 269B and 269Bv was inoculated into each of 45 Durham fermentation tubes of lactose broth containing Andrade's indicator. These were incubated at 37 C. All lactose tubes of the variant culture 269Bv showed abundant acid and gas formation in 18 hours. Of the 45 tubes inoculated with the *mutabile* strain 269B, none showed an acid reaction earlier than 48 hours, but all eventually gave fermentation of the lactose. Ten of the tubes gave a definite red color of the indicator in 48 hours; 9 required 69 hours; 4 required 77 hours; 11 required 93 hours; 6 required 117 hours; and in 5 tubes an acid reaction appeared first after 7 days. Gas formation was irregular but eventually occurred to some extent in all tubes. This result is entirely consistent with the behavior in lactose broth of the many *mutabile* strains of *E. coli* that have been studied in this laboratory. These tubes showed also another characteristic that we have found consistently in lactose broth cultures of this type of organism, namely, a reversal of the reaction to alkalinity when incubation is continued for several days or weeks. Generally the tubes that are the latest in developing acidity are the first to return to an alkaline reaction. The very earliest to become acid often do not reverse their reaction at all. The lactose broth cultures of the pure variant strains never return to a neutral or alkaline reaction.

Cultures 269B and 269Bv were also inoculated on Endo plates, 0.1 ml of the suspension being spread over the surface by means of a bent glass rod. Both plates showed many well-separated colonies after 24 hours at 37 C. The 269Bv colonies were all of the typical *E. coli* type, red with a metallic sheen. On continued incubation no daughter colonies appeared. The 269B colonies were all colorless in 24 hours, but in 48 hours a few white daughter colonies were observed which later turned red. Transplants from them to other Endo plates

gave many typical red *E. coli* colonies in 24 hours. Transplants from these colonies on still other Endo plates gave only red colonies. Tests similar to the foregoing were later made with strain 269A, which showed itself to be in all respects similar to 269B.

From all 3 strains infusion agar and lactose agar plates were made. Visible colonies developed promptly on all these plates, appearing at some time between the seventh and the twentieth hours of incubation at 37 C. All the colonies were of the smooth, moist, and round types with entire edges. There was no evidence that any S \rightarrow R variation had occurred during their long growth without transfer. A number of the colonies were inoculated into broth tubes in which they grew with even clouding, which persisted for several days without clumping. All 3 strains were later shown to be methyl-red-positive, Voges-Proskauer-negative, and indole-positive.

Since all the foregoing subcultures were made from the suspensions of the old cultures within 1½ hours of the time the suspensions were made, it must be assumed that each subculture and each colony represented the progeny of one or more living organisms in the original cultures that were capable of germinating and multiplying on the particular medium. It is clear, therefore, that in these 14-year-old agar slant cultures there were viable organisms that were capable of germinating on a variety of media and of exhibiting the same characteristics of growth and variation as their progenitors. No definite expression can be made of the number of such surviving organisms, but the numbers of colonies appearing on the plates indicated that the cultures were not yet nearing the limit of their longevity.

One is led to speculate upon the exact present state of the individual living organisms in these old cultures and upon their past history. Were the viable ones all included among the intact forms seen in stained preparations, or were some of the shadowy forms also capable of germination, or were even some of the minute particles that exhibited active Brownian movement in dark-field preparations viable? Were the viable organisms cells which have lain dormant for many years, perhaps since a few days or a few weeks after the primary phase of growth occurred, or were they merely the currently surviving cells in a culture in which continuous or intermittent multiplication had been going on accompanied by continuous death? Would these living cells, if the cultures had been left undisturbed, soon either have died or have given rise to new cells by division? There seems to be no way to arrive at conclusive answers to these questions, but the following observations are suggestive.

From one of the cultures of 269A, which had not been opened, an amount which could be picked up by merely touching the growth with an inoculating needle was placed in 10 ml of broth and thoroughly dispersed in the fluid by shaking. With sterile broth decimal dilutions of this suspension were made which were used for making plate counts in beef infusion agar. From the counts thus obtained it was calculated that the number of live organisms in the minute amount of culture used was approximately 25,000, a figure which seems reason-

ably consistent with the thesis that the intact bacilli seen in stained smears represented the viable organisms. At least this result makes it unnecessary to assume viability of any of the shadowy forms or granules.

No standard method has been devised for demonstrating the degree of dormancy or latency of bacterial cells. In fact, the existence of such a state among nonsporeforming bacteria can scarcely be said to have been proved. The most widely cited evidence for the existence of a condition of dormancy is the work of Burke, Sprague, and Barnes (1925), who found that 15 per cent of single cell transplants from 24-hour cultures of several nonsporeforming organisms, including *E. coli*, required longer than 48 hours to germinate. A few of their cultures showed their first growth as late as 16 days after inoculation. Delayed germination of individual cells in our old cultures was tested for in two ways. First, certain of the plates made for the purpose of counts were set aside for this purpose. Each colony that was visible on these plates at the end of 24 hours was marked with an ink spot. The plates were then reincubated and examined for new colonies daily for 3 weeks. After the first 48 hours the plates were placed

TABLE 1

Time required for development of visible growth in broth tubes presumably inoculated with single cells

CULTURE	MEDIUM USED	NO. TUBES INOCULATED	NO. SHOWING GROWTH IN 24 HOURS	NO. SHOWING GROWTH IN 3 WEEKS	NO. REMAINING STERILE FOR 3 WEEKS
269 A ₁	Lact. broth	51	17	17	34
269 A ₁	Inf. broth	21	8	8	13
269 B ₂	Gluc. broth	214	101	101	113

in a moist chamber to prevent drying. Not a single new colony was observed on these plates after 24 hours. Second, germination in broth was tested as follows: From the highest serial dilutions of the suspension made from the old culture a large number of infusion broth and glucose and lactose extract broth cultures were made by inoculating each with a single 4-mm loopful of the diluted suspension. If most of the tubes inoculated from one dilution gave growth while some failed to grow, it seemed reasonable to assume that most of those showing growth received an inoculum containing only one viable organism. These tubes were incubated at 37 C for 3 weeks. Again, not a single tube, not showing growth at the end of 24 hours, developed growth during this period of incubation. Table 1 gives the details of this experiment.

These results strongly suggest that the viable organisms in these old cultures consisted of structurally intact cells that were capable of prompt germination when placed in a new and favorable environment.

In conducting the foregoing experiments it was necessary to use up practically all of the growth on several of the old cultures. It was observed that when these denuded slants were stored further at room temperature they became covered with a new growth in the course of a few days. The organisms constituting this new growth proved to be neither contaminants nor variants but were identi-

cal with the original ones. After this growth had been permitted to develop for a week, it was removed as completely as possible with a loop and the slants were again stored at room temperature. No further discernible growth appeared on them during the succeeding 6 weeks. These observations show that there remained in the culture medium, after 14 years of storage, ample nutrient material for growth, and that inhibitory substances were not present in significant amount. That the fresh growth resulted in the formation of inhibitory substances is, of course, not surprising, since it has been shown by many, recently by Wheeler and Stuart (1937), that many bacterial species, including *E. coli*, form antagonistic substances during their period of rapid growth. It would appear then that such antagonistic substances as had been formed earlier in the old cultures had been destroyed in storage, leaving the medium again capable of supporting growth. It was obvious, however, that rapid growth was not taking place in the old cultures before they were denuded of their bacterial substance. The most logical explanation of this fact is that the viable organisms were largely embedded in a mass of material made up of dead and partially disintegrated cells, with only these cells and the products of their autolysis as their sources of nutriment. Steinhaus and Birkeland (1939) have shown that dead bacterial cells may serve as the sole source of nutriment for the same and other species. They found that *E. coli* grew poorly on *E. coli* cells. They did not study the nutrient properties of autolyzed cells.

The number of experiments that could be conducted on these old cultures was limited by their small number and by the changed conditions resulting from the appearance of an abundant new growth when the old growth was removed. It is believed, however, that from the observations here recorded a plausible hypothesis may be constructed to explain in part the mechanism of longevity of cultures on solid media. It is offered for what it is worth.

During the early period of growth, substances are formed that exert a strong inhibitory effect and, probably, a weak killing effect. These substances are moderately stable but, in time, deteriorate to an extent that permits further multiplication. In the meantime the gradual death and disintegration of other cells results in the eventual imprisonment of most of the viable organisms in a mass of material that furnishes a poor source of nutriment. This favors longevity in that it permits new increments of antagonistic substances to diffuse out of the organism's environment to become destroyed elsewhere, and in that the poor growth-supporting qualities reduce the amount of such substances which are formed. We may presume that in some cultures a condition would be reached in which the average generation time would become very great, because of the scarcity of food materials and the presence of a low concentration of antagonistic substances, but in which cell death would occur at a very slow rate. Such a culture would retain viable cells over a long period of time. Cultures of some species would be expected to form such highly deleterious substances during their early rapid growth as to bring about complete sterilization of the culture in a few days. Others might bring about sterilization much later, chiefly through the inability of their autolytic products to support growth.

SUMMARY

Plain agar slant cultures of two strains of *Escherichia coli-mutabile* and of the lactose-fermenting variant derived from one of them contained many viable cells after 14 years' storage at room temperature. No live cells could be demonstrated that required longer than 24 hours to germinate. No evidence of dissociation was observed in the subcultures. All three strains had retained their important biochemical characters and their ability, or lack of ability, to produce variants on lactose media. The probable mechanism of longevity of cultures on solid media is discussed briefly.

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NOTES

THE SUBSTITUTION OF THIAMINE FOR THE LABILE FRACTION OF RED BLOOD CELL EXTRACT IN THE GROWTH OF *BACTERIUM TULARENSE*¹

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In the course of a study of the red blood cell extract required for the growth of *Bacterium tularense* (Tamura and Gibby: J. Bact., 45, 361) it has been found that the activity is destroyed by heating at 120 C for 1 hour in 0.08 N NaOH,

TABLE 1

The effect of the addition of thiamine to red blood cell extract treated with heat and alkali

MEDIUM	TURBIDITY (Log I ₀ /I × 100) IN 24 HOURS
1 Basal.....	2
2 Basal + 0.06 ml blood cell extract.....	60
3 Basal + 0.10 ml blood cell extract.....	78
4 Basal + 0.06 ml blood cell extract alkali-treated.....	4
5 Basal + 0.10 ml blood cell extract alkali-treated.....	4
6 Basal + 0.4 micrograms thiamine HCl.....	2
7 As 2 + 0.4 micrograms thiamine HCl.....	102
8 As 3 + 0.4 micrograms thiamine HCl.....	105
9 As 4 + 0.4 micrograms thiamine HCl.....	101
10 As 5 + 0.4 micrograms thiamine HCl.....	102

Basal medium:

Hydrolyzed vitamin-free casein.. 1.0 per cent

Glucose..... 0.5 per cent

K₂HPO₄..... 0.01 per cent

Cysteine HCl..... 0.1 per cent

NaCl..... 1.0 per cent

pH 6.8

10 ml of medium were placed in each 180-ml pyrex bottle, autoclaved, inoculated with approximately 10⁶ washed cells per ml and incubated at 37 C with shaking.

but not by similar treatment with 0.08 N HCl. The activity removed by the alkali treatment may be completely restored by the addition of thiamine (table 1). Indeed, optimal activity is obtained at a lower level of red blood cell extract with added thiamine. In a basal medium improved by the addition of inorganic salts (Mg, 0.0002 M; Fe, 0.0000001 M; Mn, 0.0000001 M; and Ca, 0.0002

¹ Studies conducted at Camp Detrick, Frederick, Maryland, from March, 1945, to July, 1945.

² Maj., AUS.

m) thiamine alone gave in 24 hours 30 to 35 per cent of the growth produced in 24 hours by the red blood cell extract and thiamine. This confirms the observation of Berkman and Koser (J. Bact., 41, 38) that slow growth can be obtained in a gelatin hydrolyzate medium with thiamine diphosphate alone and indicates that the alkali-stable portion of the extract is stimulatory, not essential. Attempts to substitute for the alkali-stable fraction with growth factors which are alkali-stable or have alkali-stable components of known activity were unsuccessful. Pantothenic acid, nicotinamide, biotin, pyridoxine, *Lactobacillus casei* factor, *para*-aminobenzoic acid, inositol, choline, uracil, adenine, and guanine, in a mixture, or with each omitted in turn, have no effect.

A READILY PREPARED MEDIUM FOR THE CULTIVATION OF THE LACTOBACILLI

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In an attempt to correlate *Lactobacillus* counts using the dilution and plating methods, whey and tomato juice agars failed to give consistent results. Therefore, an attempt has been made to prepare a satisfactory medium from readily available dehydrated ingredients. The medium devised, which has been called "trypticase sugar agar," has the following formula:

2.0 per cent pancreatic digest of casein (Baltimore Biological Laboratory)
0.5 per cent lactose cp
0.5 per cent glucose cp
0.5 per cent sucrose cp
0.25 per cent gelatin
1.0 per cent agar

Adjust to pH 6.0 with N/1 HCl before adding agar and gelatin.

Dissolve, tube, plug, and autoclave at 15 pounds (121 C) for 15 minutes.

This medium has proved on repeated tests to be much better than the commercially available whey and tomato juice agars. Many more colonies are obtained and they are larger and more easily identified than on other media used in this study. Typical *Lactobacillus* colonies may be counted after 48 hours.

Various combinations of ingredients were tried in arriving at the present formula. The addition of soybean peptone (0.1 per cent) caused the colonies to be larger, but the plate count as compared to the regular trypticase sugar agar was somewhat less. The addition of stimulating materials such as yeast extract, liver extract, and cystine to the medium may increase the size of the colonies. However, the number of colonies is not significantly increased and may be materially decreased, and for that reason the simpler formula is considered more

desirable for routine plating. In this study a double-pour-plate technique has been used. This consists of making the usual pour plate, which is covered, after hardening, with approximately 15 ml of the same kind of agar used in pouring the plates.

A NEW SALMONELLA TYPE: SALMONELLA GATUNI

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Salmonella gatuni was isolated² from a stool specimen of a waitress at Fort Davis, C. Z. The culture was isolated April 5, 1945, and subsequent cultures taken April 15, 16, 17, 18, and May 24 were negative for organisms of the *Salmonella* group. No clinical data are available as to the course of the infection.

Cultural characteristics. The organism possesses the morphological, cultural, and biochemical characteristics of the *Salmonella* genus. It is a gram-negative, motile, nonsporeforming rod. Acid and gas are produced in glucose, maltose, mannitol, xylose, arabinose, dulcitol, rhamnose, and sorbitol. Lactose, sucrose, inositol, and salicin are not fermented. Dextro-tartrate and citrate are utilized. The organism produces hydrogen sulfide but does not form indole or liquefy gelatin.

Serological study. Alcohol-treated suspensions of the organism are agglutinated by *Salmonella oranienburg* O serum (VI, VII...) and by *Salmonella newport* O serum (VI, VIII...), which places the strain in group C according to the Kauffmann-White diagnostic schema. When tested with single factor absorbed serums, it was found to possess somatic antigens VI, VIII... .

Examination of the flagellar antigens revealed the organism to be diphasic. Phase 1 of *S. gatuni* is flocculated to the titer of serum derived from phase 1 of *Salmonella paratyphi* B (b) and in absorption tests it removes all of the flagellar agglutinins from *S. paratyphi* B serum. Phase 1 of *S. gatuni* is, therefore, designated as b.

Phase 2 of *S. gatuni* is agglutinated by *Salmonella abortus-equi* (e,n,x...) serum. Absorption tests show that *S. gatuni* will absorb all of the flagellar agglutinins from *S. abortus-equi* serum. Therefore, phase 2 of *S. gatuni* should be designated as e,n,x... .

SUMMARY

A new *Salmonella* type isolated from a human stool specimen is described with an antigenic structure that has not been previously described. This organism has the antigenic formula VI, VIII... : b; e,n,x... and is designated *Salmonella gatuni*.

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² Isolated by Kurt F. Mensel, Senior Medical Technician, Gorgas Hospital, Ancon, Canal Zone.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND EIGHTY-FOURTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY
BUILDING, PHILADELPHIA, PENNSYLVANIA, JANUARY 22, 1946

CHOLERA IN CHUNGKING IN 1945: USE OF STREPTOMYCIN. *Hobart A. Reimann*, Jefferson Medical College, Department of Medicine, Philadelphia, Pennsylvania.

About 2,500 cases of cholera were recorded in Chungking in the summer of 1945, but many more unrecognized and unrecorded cases no doubt occurred. From several patients, *Vibrio comma* of the Ogawa type was recovered. In a series of 160 patients treated vigorously by controlled rehydration at the Shao Lung Kan Hospital, the mortality rate was 5 per cent, as compared with 16 per cent among those less thoroughly managed elsewhere. Ten patients were given streptomycin orally, 4 grams daily, and a few 3 to 4 grams parenterally in addition, but other than an apparent slight shortening of the disease and disappearance of vibrios in stained films from stools, no beneficial effects were noted. *V. comma* could be cultivated from the stools during treatment in all. Strains of *V. comma* from this epidemic varied greatly in their sensitivity to streptomycin. Some were inhibited by only 5 micrograms, others resisted 500 micrograms.

Evidence suggested that the epidemic did not arise or spread chiefly from a polluted public water supply, but more likely was perpetuated by many errors in personal hygiene. Cholera will persist in endemic areas where there are ignorance, poverty, and overcrowding, but disappears spontaneously when the standard of living improves. Much can be done to check an epidemic by educational propaganda alone. The control of cholera is more of an economic problem than a medical one.

THE SHADOW-CASTING TECHNIQUE IN ELECTRON MICROSCOPY. *Thomas F. Anderson*,

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The technique of oblique evaporation of metal onto electron microscope specimens as developed by Williams and Wyckoff (Science, 101, 594) gives pictures of such excellent three-dimensional quality that in the future most specimens will probably be examined by this technique. Other advantages are that one can infer the shape of an object from the distribution of metal deposited upon it and from the shape of the shadow which the object casts on the supporting material. Likewise, the increased contrast of minute objects coated with films of a heavy metal such as gold makes them distinctly visible for the first time.

A device was described which permits one to evaporate metal onto a specimen while it is in position in the electron microscope itself, thus saving time and avoiding damage to the specimen in unnecessary handling. The advantages of the shadow-casting technique in general were illustrated by micrographs of bacteriophage, cells of *Escherichia coli*, and of collagen.

PHASE MICROSCOPY. *Oscar W. Richards*, Research Division, American Optical Company, Scientific Instrument Division, Buffalo, New York.

Many microscopical specimens of biological and industrial importance are so transparent that the details of their structure cannot be seen by ordinary bright-field microscopy. The phase microscope makes visible such detail when it differs from its surroundings either in thickness or index of refraction for light, i.e., in optical path. The differences in the phase of the light passing through the specimen are changed to intensity differences, which are visible and may be photographed, in the

phase microscope with a diffraction plate introduced into the objective. The contrast in the image, formed by the microscope, may be reversed, increased, or re-

duced. Applications were illustrated, including bacteria, tissue cultures, crystals, protozoa, emulsions, and transparent surfaces.

INDIANA BRANCH

INDIANAPOLIS, INDIANA, FEBRUARY 8, 1946

PREPARATION OF JAPANESE TYPE "B" ENCEPHALITIS VACCINE. *LaVeda Stanfield and Lorraine Myers*, The Lilly Research Laboratories, Indianapolis, Indiana.

Since the antigenic substance in Japanese B encephalitis vaccine is derived from mouse brains which have been infected with the Nakayama strain of Japanese B encephalitis virus, it was important to use mice whose brains would yield an intracerebral titer of 10^{-8} or greater.

Comparative tests were made using several strains of Swiss mice in order to find a strain or strains capable of meeting this qualification. Variations in susceptibility to the virus were encountered; consequently those strains which did not produce the desired titer were eliminated.

Lots of vaccine processed from virus suspensions, which had had an intracerebral titer of greater than 10^{-8} before inactivation of the virus, had LD_{50} doses of as low as

0.002 ml. Japanese B encephalitis vaccine cannot be released for use if the LD_{50} dose is greater than 0.01 ml.

NEW METHOD OF TESTING ANTIRABIC VACCINE IN WHITE MICE. *Mary Ann Kibler*, Biological Processing Laboratory, Eli Lilly and Company, Indianapolis, Indiana.

Not so long ago, the potency of rabies vaccine was determined by evaluating the M.L.D. of the living virus. Since this method did not prove the immunity produced by devitalized virus, a mouse protection test developed by Habel has been used in determining the potency of all vaccine produced in this laboratory for the past two years. The results of this test are determined by computing the LD_{50} of the immunized and control mice according to the Reed-Muench method, and each batch of vaccine distributed must show protection against at least 1,000 M.L.D.'s, as determined by this method.

SCIENTIFIC PROCEEDINGS

FORTY-SIXTH GENERAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

DETROIT, MAY 21 TO 24, 1946

ABSTRACTS¹

Division of General Bacteriology

- | | |
|--|-------------------------------------|
| G 1-6. Test Methods and Miscellaneous. | G 36. Protein Metabolism and Im- |
| G 7-12. Physiology. | munity. |
| G 13-23. Nutrition and Metabolism. | G 37. Physical Methods and Bacteri- |
| G 24-30. Taxonomy. | ology. |
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Division of Agricultural and Industrial Bacteriology

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|--------------------------------|---|
| A 1-8. Sanitary Bacteriology. | A 27-32. Miscellaneous Studies. |
| A 9-17. Antibiotics. | A 33-41. Disinfection and Chemotherapy. |
| A 18-26. Industrial Processes. | |

Division of Medical Bacteriology, Immunology, and Comparative Pathology

- | | |
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| M 1-5. Antibiotics: streptomycin. | M 29-36. Immunization Problems. |
| M 6-15. Miscellaneous Studies. | M 37-44. Comparative Pathology. |
| M 16-28. Viruses. | M 45-51. Miscellaneous Studies. |

Round Tables and Symposia

- | | |
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| RT 1-6. Problems of Teaching. | RT 26-31. Taxonomy. |
| RT 7-14. Michigan History. | RT 32-33. Food Processing. |
| RT 15-21. Influenza. | RT 34-38. Air Disinfection. |
| RT 22-25. Streptomycin. | RT 39-46. Immunizing Fractions. |

G1. The Development of Quick Microtechniques for the Identification of Cultures.

R. H. WEAVER, W. M. ARNOLD, JR., AND JOHN HANNAN, University of Kentucky, Department of Bacteriology, Lexington, Ky.

The use of small tubes, large inocula, and preheating of media makes it possible to determine the biochemical characteristics of bacteria quickly. An indole test, based on these principles, has yielded positive results, in some cases, after six minutes' incubation. It gives reliable results with members of the *Enterobacteriaceae* and with some other organisms within approximately two hours. Acid and gas production from glucose has been obtained in less than one hour, although it usually requires between one and two hours. The disaccharides are fermented somewhat more slowly. Other tests are under study. It is hoped that a system of techniques can be developed by the use of which it will be possible

¹ This number of the Journal has been edited by the Chairman of the Program Committee. Authors of abstracts have not seen proof due to restrictions of time imposed by the printing schedule.

tentatively to identify bacteria which multiply quickly within a few hours after their isolation.

G2. Applicability of Modifications of the Thin Filter Paper Disc Method to the Assay of Enzymes. MARION B. SHERWOOD, The Wellcome Research Laboratories, Department of Bacteriology, Tuckahoe 7, N. Y.

That the uniform absorptive capacity of filter paper discs renders them useful for assay work in fields other than antibiotic testing has not been generally recognized. Under conditions similar to those developed for penicillin assay the logarithmic dose-response relationship between amylases from different sources and various kinds of starches is readily demonstrable. The assay of starch-splitting enzymes including purified preparations of α and β amylase will be discussed with reference to the establishment of the optimum conditions for testing. Preliminary work upon proteinases and other types of enzymes for which concentration-response curves have been established will also be presented.

G3. Methods of Study of Antiphage Agents Produced by Microorganisms. DORIS JONES AND ALBERT SCHATZ, Rutgers University, Department of Microbiology, New Brunswick, N. J.

For the purpose of testing large numbers of cultures of microorganisms for their ability to produce agents active against viruses methods similar to those used in the study of antibacterial substances were used, namely, the phage agar plate, phage agar streak, and the agar diffusion or cup test methods.

The fundamental principle in all these methods involves the growth of the antagonist upon, or the diffusion of the active agent into, phage-seeded agar, followed by flooding the surface with host-seeded agar. Antiphage action is indicated by a reduced number of plaques or by a zone of bacterial growth surrounding either the antagonist or the cup containing the active substance. The rest of the plate shows complete lysis of the host cells by the uninhibited phage.

The methods used for the isolation and study of antiphage agents inherently possess greater variability and difficulty than the analogous procedures used in the study of antibiotics. This is due to the increased complexity of the biological system by the addition of bacteriophage. It remains to be determined to what extent these techniques, or certain modifications thereof, can be utilized for the isolation and study of agents active against plant and animal viruses.

G4. Aeration in the Cultivation of Brucella suis. PHILIPP GERHARDT, 1ST LT., INF., AND LYNN L. GEE, 1ST LT., AC., Camp Detrick, Frederick, Md.

The aeration requirements of *Brucella suis* in broth culture have been studied with a view to maximum production of cells in a minimum growth period. Air was supplied to the substrate either through the turbulence of shaken flasks containing relatively small amounts of medium or through the dispersal of air by various types of spargers into deep quantities of medium. A tryptose broth medium, fortified with glucose and thiamine, was employed.

Experience gained during the investigation indicated that aeration was as critical a limiting factor in maximum growth of the organism as nutrients, inocula, or incubation temperature. With the extent of growth of the organism as the criterion, optimum conditions were established for shaken flasks and sparger aeration systems. Employment of a properly constructed reciprocal shaker provided a high, yet constant and accurately reproducible rate of aeration for the growing cultures, thus making the apparatus particularly valuable for the study of other variables where aeration must be constant. Employment of sparger aeration systems provided an effective method for laboratory production of comparatively large amounts of cells, if efficient types of spargers, adequate rates of aeration, and suitable antifoam agents were used. With either of these systems, the normal growth cycle of the organism was reduced from 50-70 hr to less than 24 hr, while cell yields were increased from less than 10 billion bacteria per ml to more than 50 billion bacteria per ml.

G5. A Critical Evaluation of the Nitrogen Assimilation Tests as Commonly Used in the Classification of the Yeasts. LYNFERD J. WICKERHAM, Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill.

The ability of certain closely related groups of yeasts to utilize nitrate nitrogen for growth has proved to be a valuable characteristic in classification. For the past 12 years the ability to assimilate ammonium sulfate, urea, asparagine, and peptone has also been widely adopted in taxonomic studies. Most nonsporeogenous yeasts are reported to utilize all of these four nitrogen sources, prominent exceptions being the members of the genus *Kloeckera* and five species of *Torulopsis*. Monographs on the *Candida* genus by the different workers have shown marked variations in urea utilization within the same species. All used the auxanographic plate technique with only minor variations in the medium. The medium consisted of glucose, potassium phosphate, magnesium sulfate, agar, and the nitrogen compound under test.

By additions to this medium of pure vitamins, trace elements, and the salts calcium chloride and sodium chloride, all yeasts studied were able to utilize ammonium sulfate, urea, asparagine, and peptone. Included were four of the five species of *Torulopsis* previously reported incapable of utilizing ammonium sulfate, urea, and asparagine; three species of *Kloeckera*; and all species of the *Candida* genus previously reported negative for urea. To obtain growth with all strains in urea, it was necessary to use a concentration of approximately 0.05 per cent or less of this substance.

It is thus apparent that most, if not all, of the previously reported inability of certain yeasts to utilize ammonium sulfate, urea, and asparagine are in error owing to the poor nutritional quality of the medium used in these studies.

G6. The Viability of Heat-activatable Spores in Distilled Water or Glucose Solution as Influenced by Prestorage or Poststorage Heating. HAROLD R. CURRAN AND FRED R. EVANS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington 25, D. C.

Changes in the apparent viability of heat-activatable spores were observed in two separate fluid mediums: in one the cells were provided a source of energy but no nitrogen, the other providing neither nitrogen nor a source of energy. Exhaustively washed spores of a heat-activatable strain of *Bacillus subtilis* (LB) were suspended in glucose solution (0.1 or 1.0 per cent, Baker's cp) or in distilled water. Plate counts on glucose tryptone agar were made before and after heating at 95 C for 15 minutes and again after storage at 37 C for varied periods with and without poststorage heating at 95 C for 15 minutes. In distilled water without prestorage heating, there was no significant change in the number of spores that produced colonies over a period of one to two months. With prestorage heating, most of the spores germinated within a month, but poststorage heating of this suspension materially increased the count. In glucose solution without prestorage heating, the count decreased progressively with the length of the storage period; with prestorage heating, the vast majority of spores became unable to form colonies after two days of storage, the count declining further with the time of storage. Poststorage heating of the latter sample materially increased the count. Other brands of glucose and an especially purified preparation yielded similar results. One per cent was only slightly less effective than one-tenth per cent glucose in reducing the count. A provisional interpretation of results is made based in part upon the microscopical examination of stained and unstained spore suspensions at different stages of storage.

G7. *Production of Vitamin B₂ by Mycobacterium smegmatis.* R. L. MAYER AND MARCELLE RODBART, Ciba Pharmaceutical Products, Inc., Summit, N. J.

Mycobacterium smegmatis produces, when grown in Long's medium, varying amounts of vitamin B₂ (up to 135 µg/ml, or 36 mg/g dry bacteria), as identified by biological and physicochemical methods. The different environmental factors influencing its formation have been investigated. A study of nitrogen sources showed that the greatest amounts of vitamin B₂ are formed if only inorganic nitrogen is present; the presence of organic nitrogen (for example, asparagine) results in increased growth, but decreased vitamin B₂ production. The influence of the various carbon sources is more complex. Citrate appears to effect chiefly the rate of growth, while glycerine chiefly effects the amount of vitamin produced. Glucose can replace glycerine as the carbon source, but only if asparagine also is present. Greater yields of vitamin B₂ are obtained when glycerine is replaced by certain sugars, such as arabinose or levulose. Citrate as a growth factor can be replaced by various acids. The amount of magnesium and potassium in the medium proportionately influences the growth and vitamin production, whereas sodium and chlorine have no effect. The influence of iron was slight. The optimum pH was found to be about 6.0.

G8. *Influence of Iron Concentration and Attenuation on the Metabolism of Clostridium acetobutylicum.* AUSTIN M. HANSON AND NELSON E. RODGERS, Western Condensing Company, Research Laboratories, Appleton, Wis.

A study of the fermentation of whey by a derived strain of *Clostridium acetobutylicum* revealed a relation between iron concentration and distribution of products and pronounced differences in the metabolism of "normal" and "attenuated" strains. The basal medium consisted of rennet whey containing about 4×10^{-6} M iron supplemented with 2×10^{-5} M ZnSO_4 , 2.5×10^{-5} M MnSO_4 , 10 parts per billion *p*-aminobenzoic acid, 0.15 per cent CaCO_3 , 0.15 per cent $\text{Ca}_3(\text{PO}_4)_2$, and iron as indicated. The inoculum for the normal culture was taken from the fifth transfer in the basal medium supplemented with 2×10^{-5} M iron. The attenuated strain was derived from the normal culture by 50 transfers at 24-hour intervals in the same medium. The fermentations were analyzed after 38 hours' incubation at 37 C. When the normal culture was tested in the presence of 0.75, 2.0, and 8.0×10^{-5} M iron, lactate, formate, and ethanol production per unit of sugar fermented diminished as the iron concentration was increased; whereas hydrogen, carbon dioxide, acetone, acetoin, butyrate, and acetate production increased with iron concentration. Butanol production was not affected by iron concentration. Carbon recoveries and oxidation-reduction balances were good. The attenuated strain grown in the presence of 2×10^{-5} M iron fermented sugar very incompletely, and lactate was the principal product.

These findings parallel the observations of high lactate production by *Clostridium perfringens* (Pappenheimer and Shaskan, 1944) and *Aerobacter indologenes* (Waring and Werkman, 1944) when grown in iron-deficient media.

G9. Recovery of Biotin from Cultures of Acetone, Butyl Alcohol Bacteria. ROSARIO REYES-TEODORO AND MILO N. MICKELSON, University of Michigan, Department of Bacteriology, Ann Arbor, Mich.

An attempt was made to determine the fate of biotin added to a synthetic medium in which were cultured acetone, butyl alcohol bacteria, for which this vitamin is an essential growth factor. Assays of the bacterial cells and the cell-free medium for biotin were carried out using the titrimetric procedure of Shull, Hutchings, and Peterson. Four methods were employed to liberate biotin from the cells: (1) autolysis, (2) hydrolysis with hydrochloric acid, (3) digestion with papain, and (4) digestion with a mixture of papain and takadiastase.

From the data obtained the following findings seemed evident: (1) that the maximum biotin recovery amounted only to 75 to 80 per cent of the quantity added to the medium; (2) that about 15 to 20 per cent of the biotin recovered was present in the medium and the remainder was extracted from the cells; (3) that about 20 to 25 per cent of the biotin could not be recovered at all; and (4) that acid hydrolysis and digestion with both papain and takadiastase simultaneously offer the best methods of extracting biotin from the bacterial cells.

G10. Relation of Strain Variation and Culture History to the Synthesis of Riboflavin by *Clostridium acetobutylicum* in Whey. NELSON E. RODGERS, RICHARD H. HENIKA, AND AUSTIN M. HANSON, Western Condensing Company, Research Laboratories, Appleton, Wis.

The production of practically significant quantities of riboflavin by fermentation of whey with *Clostridium acetobutylicum* is dependent largely on selection of suitable strains, maintenance of culture stability, and adjustment of the iron concentration in the medium to a level optimum for growth but not inhibitory to riboflavin synthesis. Because of its exceptionally low iron content (about 0.2 ppm) a suitably supplemented whey is an excellent medium for the synthesis of riboflavin. However, since iron contamination is difficult to control in large-scale operations, it is desirable to select strains not only for superior riboflavin production, but also for a high threshold of inhibition of riboflavin synthesis by iron.

Tests on 13 strains revealed iron optima ranging from 0.5 to 1.6 ppm and riboflavin yields of 8 to 23 μg per ml. Modification of these strains by special culture treatment resulted in iron optima of 1.0 to 1.6 ppm and riboflavin yields of 18 to 78 μg per ml. Although in most cases increased iron tolerance was associated with increased riboflavin synthesis, two cultures showed improved synthesis unaccompanied by a change in iron optimum, suggesting that the two characters are not genetically linked.

The capacity to synthesize riboflavin is an extremely unstable character and is attenuated rapidly by serial transfer of aged cultures. In the development of inocula for large-scale fermentations, attenuation can be prevented by adjustment of the transfer schedule to fit certain physiological phases of the culture cycle.

G11. *The Amino Acid Composition of Microorganisms.* J. L. STOKES AND MARION GUNNESS, Merck and Co., Inc., Microbiological Research and Development Department, Rahway, N. J.

The quantities of ten amino acids, namely, histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, and tryptophane, were determined microbiologically in the acid or alkaline hydrolyzates of the dried cells of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Streptomyces griseus*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, *Rhizopus nigricans*, *Aspergillus niger*, and *Penicillium notatum* grown under a variety of cultural conditions. The amino acid composition of an organism is, qualitatively and quantitatively, a stable and characteristic property of the cell under fixed conditions of growth. Although striking quantitative differences occur between microorganisms, the results, in general, emphasize the similarities rather than the differences in their amino acid composition. Certainly no fundamental differences in that some amino acids are present in one organism but not another were encountered. The microbial proteins do not appear to differ materially from plant and animal proteins represented by wheat and beef liver. Fungi contain 10 to 50 per cent less, per unit of protein, of most of the amino acids than the other microbial groups. Mold mycelium prior to sporulation compared to that after sporulation contains considerably larger quantities of most of the amino acids largely because of its 50 per cent greater protein content. The mycelium and its spores, in general, have comparable amino acid contents. The quantities of individual amino

acids in microorganisms may vary with the growth medium, aeration, and age of the cells.

G12. The Amino Acid Composition of Crystalline Botulinus (Type A) Toxin.

HENRY J. BUEHLER, D. H. BORNOR, E. J. SCHANTZ, AND CARL LAMANNA,
Camp Detrick, Frederick, Md.

A method for isolation and crystallization of pure botulinus toxin, type A, has been reported. In preliminary studies of this material the presence of 14 amino acids has been determined. The toxin appears to be a complete protein containing the 10 essential amino acids necessary in animal nutrition. There is no suggestion of an unusual amino acid composition that might be used to explain the extreme toxicity (32 billion mouse LD₅₀ per g of dry weight). As with diphtheria toxin the aromatic amino acids appear to be present in abundance. A dicarboxy amino acid, glutamic acid, is present in greatest amount.

Positive qualitative chemical tests have been obtained for arginine, lysine, histidine, threonine, and tryptophane. Negative tests have been obtained for glycine, alanine, proline, and hydroxyproline. Positive tests have been obtained for free sulfhydryl groups in the whole protein.

Microbiological assay was finally chosen as the most practical and economic technique for quantitative study of amino acid content of the toxin. These assays have confirmed chemical tests for presence of arginine, lysine, histidine, and threonine. The following amino acids have been shown to be present: aspartic acid, valine (4.0 per cent), serine (3.0 per cent), threonine (7.3 per cent), arginine (3.2 per cent), lysine (7.9 per cent), histidine (1.0 per cent), glutamic acid (14.9 per cent), leucine (8.7 per cent), isoleucine (11.0 per cent), cystine (0.48 per cent), methionine (0.83 per cent), phenylalanine (1.08 per cent), tyrosine (9.5 per cent). The quantitative data listed are not to be considered final.

G13. Nutritional Studies with Clostridium botulinum, Toxin Types A and B.

W. G. ROESSLER, ENS., USNR, AND C. R. BREWER, Camp Detrick,
Frederick, Md.

The nutritional requirements of *Clostridium botulinum*, toxin types A and B, for growth and toxin production were studied. Experiments were designed to test the effects, interrelationships, and requirements of amino acids, vitamins, nucleic acid components, and salts. Washed spores were used as the inoculum, and cultures were grown in Brewer or Fildes jars incubated at 35 C. The Coleman photoelectric colorimeter was used to evaluate the growth obtained. All toxin tests were performed on mice. Following the development of a gelatin hydrolyzate medium, amino acid studies were made in chemically defined media. The following amino acids are essential for the growth of both types: leucine, isoleucine, valine, arginine, phenylalanine, tryptophane, tyrosine, and methionine. In addition, threonine is essential for the growth of type A and histidine for type B. Nucleic acid or its components are necessary for good growth and toxin

production of type A. With type B, nucleic acid is less important. Biotin is the only essential vitamin for both types; however, better growth occurs if riboflavin, *para*-aminobenzoic acid, and niacinamide are included. Type A utilized desthiobiotin in place of biotin but type B did not. Calcium pantothenate, pyridoxine, thiamine, inositol, and folic acid are not required for growth by either type. Toxin titers in excess of 10^8 mouse MLD/ml with type A and 5×10^8 mouse MLD/ml with type B were obtained.

G14. *The Nutrition of Coccidioides immitis in Submerged Culture.* W. G. ROESSLER, ENS., USNR; E. J. HERBST, ENS., USNR; W. G. McCULLOUGH, 1ST LT., AUS; R. C. MILLS, ENS., USNR; AND C. R. BREWER; Camp Detrick, Frederick, Md.

Coccidioides immitis, a virulent fungous pathogen, has previously been grown as a surface mat on solid or liquid culture media. In order to obtain greater yields, to eliminate the hazard of working with dry spores, and to permit more accurate evaluation of results, culture techniques and media suitable for submerged growth were developed. Dilute spore suspensions were used as inocula. Cultures were grown in pyrex flasks. Aeration was provided by continuous mechanical agitation which also aided in the disintegration of sporulated mycelia to form free spores. Results were evaluated by poured plates. The nutritional requirements of *C. immitis* are relatively simple, as has been reported by other workers. Yields consisting mainly of spores, occurring singly and in short chains, in excess of 2×10^8 viable fragments per ml were obtained in chemically defined media of the following composition: 0.08 M ammonium acetate, 0.11 M glucose, 0.008 M $MgSO_4$, 0.015 M K_2HPO_4 , 0.015 M KH_2PO_4 , and 0.00003 M $ZnSO_4$. Magnesium and zinc were the only metallic ions found to be required for growth. No vitamins were required. In submerged cultures the following sequence of development was observed: spore germination, growth of nonseptate mycelia, septation, rounding of septa to form spores, maturation of spores, and finally disintegration of spore chains with liberation of free spores. Maximum turbidities were attained after five days' incubation; ten days were required for maximum plate counts. The spores produced were fully virulent for guinea pigs and closely resembled *C. immitis* grown on surface cultures in morphology.

G15. *The Nutritional Requirements of Bacillus anthracis.* C. R. BREWER; W. G. McCULLOUGH, 1ST LT., AUS; R. C. MILLS, ENS., USNR; W. G. ROESSLER, ENS., USNR; E. J. HERBST, ENS., USNR; AND A. F. HOWE, 2ND LT., AUS.; Camp Detrick, Frederick, Md.

Because of scanty and conflicting information in the literature, an investigation was undertaken to determine the nutritional requirements of *Bacillus anthracis*. The object was to produce free mature virulent spores in media of known chemical composition with yields equal to those obtained in the usual natural media. The accepted microbiological nutrition techniques were used to eliminate extraneous factors as chemical impurities, carry-over of growth factors in the inocula, and uncontrolled physical environment. Cultures were grown in

pyrex bottles aerated by continuous mechanical shaking. Results were evaluated by plate counts. Yields in excess of 10^9 virulent spores per ml in a medium composed of amino acids (synthetic where available), glucose, thiamine, glutamine, uracil, adenine, guanine, bicarbonate, phosphate, and salts of potassium, calcium, iron, magnesium, and manganese. No B complex vitamins other than thiamine were required. Uracil, adenine, and guanine in combination, but not singly, stimulated growth considerably. Yields were increased greatly by adjusting the concentrations of the inorganic constituents of the media to optimal levels. The results of the fundamental nutritional studies were applied to the development of simplified natural media (tryptic digest of casein, autolyzed yeast, glucose, and salts) which have produced yields in excess of 2.25×10^9 spores per ml. These media have been valuable in immunological studies by others.

G16. Plating Media Requirements for Certain Strains of *Bacterium tularensis*.

F. B. ENGLE, JR., 2ND LT., SNC, AND T. L. SNYDER, 1ST LT., SNC, Camp Detrick, Frederick, Md.

An extensive survey was made of plating media requirements for *Bacterium tularensis* strain Schu. The basal medium consisted of bacto peptone, sodium chloride, cysteine, and glucose. This medium was altered in various ways, and the results were evaluated on the bases of rate of colonial growth and the variability among counts of replicate plates.

Bacto peptone could be replaced by proteose peptone, proteose peptone no. 2, and proteose peptone no. 3 or neopeptone, but not by bacto protone, bacto tryptose, bacto tryptone, Difco beef extract, bacto gelatin, Difco casamino acids, Wilson peptone USP, Baker peptone USP, or Sheffield peptinase. In the presence of thiamine, apparently optimum growth was obtained with bacto tryptone, but this was not true with the remaining peptones. The oxidation-reduction potential of the medium was adequately adjusted with 0.1 per cent cysteine. Freshly prepared peptone agar appeared to give more uniform results than plates stored for several days. The hydrogen-ion concentration had little effect between pH 6.5 and 7.4. The optimum sodium chloride concentration fell between 0.5 and 1 per cent. Glucose was not necessary for growth. Human erythrocytes had no certain effect other than to prevent inhibition by high concentrations of glucose. Statistical analysis of results failed to demonstrate any differences between counts on fresh peptone agar and on blood, cysteine, glucose agar.

Of 28 strains tested with the optimum medium for strain Schu, 14 appeared to give satisfactory results and 14 grew poorly with small inocula.

G17. The Reduction of Trimethylamine Oxide by Representatives of the Genus *Pseudomonas*. E. R. HITCHNER, University of Maine, Department of Bacteriology and Biochemistry, Orono, Maine.

A study has been made of the ability of representatives of the genus *Pseudomonas* to reduce trimethylamine oxide to trimethylamine. Ninety-eight strains of polar-flagellated, gram-negative bacteria, representing 15 species including *P. hydrophila* and *P. ichthyosmii* and several strains whose specific designation

was not determined, were tested according to the procedure described by Wood and Baird. The ability to ferment glucose, lactose, sucrose, glycerol, and to produce acetylmethylcarbinol from glucose was included in the study.

The aerogenic species, *P. hydrophila* and *P. ichthyosmii* and 2 strains listed as *P. putrefaciens*, formed trimethylamine, whereas all other cultures, including 2 strains listed as *P. putrefaciens*, which were nonaerogenic, failed to produce this compound. Those strains which reduced trimethylamine oxide likewise produced acetylmethylcarbinol from glucose.

These results indicate that the metabolism of the aerogenic species differs materially from the nonaerogenic strains and further indicates that the mechanism of the utilization of carbohydrates (true fermentative ability) by aerogenic strains of polar-flagellated organisms so differ from the nonaerogenic (oxidative utilization of carbohydrates) as to warrant their inclusion in the genus *Aeromonas* of the family *Pseudomonadaceae* (Kluyver and van Niel) as suggested by Stanier.

G18. The Oxidation of Glycerol by *Escherichia freundii*. M. N. MICKELSON AND F. E. SHIDEMAN, University of Michigan, Departments of Bacteriology and Pharmacology, Ann Arbor, Mich.

Relatively little information has been published on the mechanism by which glycerol is metabolized by bacteria. The present work is concerned with the role of phosphate in the oxidation of glycerol by *Escherichia freundii*. Addition of inorganic phosphate resulted in increased O_2 uptake and glycerol consumption.

The respiratory quotient in the absence of added phosphate is less than, but approaches, the theoretical for complete oxidation of glycerol. In the absence of added phosphate glycerol oxidation is 68 to 83 per cent complete, in the presence of phosphate 10 to 40 per cent complete. Addition of inorganic phosphate produces a marked decrease in the R.Q., and the low R.Q. is associated with increased glycerol utilization. 0.1 M phosphate is the optimum concentration.

There is an uptake of inorganic phosphate when glycerol is oxidized which reaches a maximum at 40 minutes, during which time O_2 uptake is linear. When O_2 uptake decreases, inorganic phosphate is released into the medium until at end of 100 minutes most of the inorganic phosphate esterified has reverted to its original form. The inorganic phosphate esterified was nearly all found in the fraction resistant to hydrolysis in normal acid at 100 C for 180 minutes.

Adenosine triphosphate appears to be about 2.5 times as efficient as inorganic phosphate. 0.075 M NaF causes inhibition of O_2 uptake in absence of inorganic phosphate but not in its presence. 0.001 M cyanide and 0.0005 M iodoacetate inhibit glycerol oxidation in presence and absence of phosphate.

G19. A Simplified Medium for the Microbiological Assay for Pantothenic Acid. HELEN M. KETCHUM AND RAYMOND L. CONKLIN, Miles Laboratories, Inc., Department of Medical Research, Elkhart, Ind.

A medium for the microbiological assay for pantothenic acid in material of high potency vitamin content is described. *L. arabinosus* is used as the test organism. It appears unnecessary to employ complicated media which can

contribute to the possibility of greater errors. By increasing the sugar and sodium acetate, as suggested by Stokes and Martin, the original formula of Pennington and Snell and Strong and his associates was found to be very satisfactory with the following modifications: peptone 1 per cent, glucose 4 per cent, sodium acetate 3.6 per cent, casein 0.4 per cent, hydrolyzed casein 0.4 per cent, cystine 100 mg, riboflavin 200 mg per L, and inorganic salts. A comparison has been made of the curves obtained with this medium in contrast to curves obtained with generally accepted media.

G20. Significance in Nutritional Research of Correct Identification of *Lactobacillus casei*, *L. delbrueckii*, and *L. bulgaricus*. MORRISON ROGOSA, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

The nutritional requirements of lactobacilli designated *Lactobacillus delbrueckii* LD5 and *L. bulgaricus* 05 have been described in the literature. Taxonomic study of these strains by the author proved definitely their identity as *L. casei*. As in the case of *L. casei*, cells are small and colonies smooth. They grow in defined media; at 10 C; in 4 per cent NaCl; and produce approximately 1.40 to 1.47 per cent acid in milk held at 30 C for two weeks. The acid is predominantly dextrorotatory. They ferment lactose, maltose, sucrose, mannitol, trehalose, salicin, sorbitol, and glycerol.

Authentic strains of *L. delbrueckii* and *L. bulgaricus* do not form smooth colonies; do not grow at 10 C; do not grow in 4 per cent NaCl; do not produce dextro acid; do not ferment mannitol, trehalose, salicin, sorbitol, glycerol. *L. delbrueckii* does not ferment lactose or curdle milk. It forms rough colonies; ferments maltose and sucrose; produces mainly levo lactic acid from glucose. *L. bulgaricus* forms rough colonies; ferments lactose but not sucrose and maltose. In milk it produces an average of 2.7 per cent lactic acid. The acid is optically inactive.

Unlike *L. casei*, neither *L. delbrueckii* nor *L. bulgaricus* grows in defined media containing all known B vitamins and amino acids. These species require an unknown factor or factors. Thus, authentic strains of *L. delbrueckii* and *L. bulgaricus* should prove useful as tools in research for unknown growth factors.

G21. Unidentified Trace Element Requirements of Photosynthetic Purple Bacteria.

S. H. HUTNER, Haskins Laboratories, New York 17, N. Y.

Following identification of the vitamin requirements of purple bacteria (kindly supplied by Professor van Niel), the inorganic requirements of *Rhodospirillum rubrum* and *Rhodopseudomonas capsulatus* (requiring respectively biotin and thiamine) were studied. The other organic constituents of the medium were redistilled ammonium acetate and propionic acid, and 0.025 per cent Na-citrate $\cdot 2\text{H}_2\text{O}$.

Virtually any assortment of elements such as Fe, Mn, Zn, Cu, Mo, etc., supported heavy growth if the total concentration exceeded approximately 0.2 mg per cent. Ash was ineffective. Many substances of biological origin (asparagine, lactate, sugars, etc.) effectively replaced these mixtures of heavy elements.

The interchangeability of these elements, and the excessive quantities required, were traced to the fact that these elements were contaminated with unidentified essential elements, and that the true requirement for Fe and other recognized essential trace elements was probably less than 0.001 mg per cent. The platinum elements, and, significantly, elements susceptible to loss by volatilization on ashing, e.g., mercury, osmium, rhenium, and ruthenium, proved exceedingly favorable when supplied in nontoxic concentrations ($< 0.001\text{--}0.1\mu\text{g}$ per cent). The partial interchangeability of these elements suggested that their activity in turn depended on additional mutual impurities.

The extreme toxicity, in precipitate-free media, of elements proving favorable in lower concentrations, was a fertile source of misleading results: many elements formed precipitates in dilute media, and thereby removed toxic elements by precipitation or adsorption, and hence would seem favorable or even essential.

G22. Fixation of Heavy Carbon Acetaldehyde by Active Juices. NOEL H. GROSS AND C. H. WERKMAN, Iowa State College, Department of Bacteriology, Ames, Iowa.

There is no general agreement as to the mechanism of the biological formation of acetylmethylcarbinol. Evidence has been presented to support acetaldehyde as an intermediate in its formation from glucose. Other evidence has been presented that acetylmethylcarbinol is formed from pyruvic acid, acetic acid, and citric acid.

Experimental data have been obtained that contribute to the clarification of the role of acetaldehyde as an intermediate in the formation of acetylmethylcarbinol. Juices prepared from *Aerobacter* did not utilize synthetic acetaldehyde in the formation of acetylmethylcarbinol. Heavy carbon acetaldehyde, with C^{13} in both positions, when added to a pyruvic acid fermentation, yielded acetylmethylcarbinol with only the normal percentage of heavy carbon.

The yeast juice enzyme preparation, on the other hand, can utilize synthetic acetaldehyde in the formation of acetylmethylcarbinol. When heavy carbon enriched acetaldehyde was added to the yeast juice fermentation of pyruvate, acetylmethylcarbinol was formed containing increased amounts of heavy carbon.

Pig heart juice appears to be quite different from either the bacterial juice or the yeast juice. This preparation formed large amounts of acetylmethylcarbinol from acetaldehyde alone. The addition of pyruvate did not increase the carbinol production.

The enriched heavy carbon acetylmethylcarbinol formed by the yeast juice was degraded and heavy carbon was found in all four carbons.

G23. Mechanism of Pyridoxal Phosphate Function in Bacterial Transamination.

W. W. UMBREIT, D. J. O'KANE, AND I. C. GUNSALUS, Cornell University, College of Agriculture, Laboratory of Bacteriology, Ithaca, N. Y.

The interconversion of pyridoxal and pyridoxamine by heating with amino and keto acids, respectively, led Snell to suggest that these substances function

in transamination, the compounds themselves undergoing transamination in the process. This view was further strengthened by a demonstration of the conversion of pyridoxamine into pyridoxal phosphate by incubation with a keto acid and *Streptococcus faecalis* cells.

The present study has shown that the glutamic-aspartic apotransaminase prepared from *Streptococcus faecalis* in a cell-free state can be activated by pyridoxamine phosphate, in addition to pyridoxal phosphate. The pyridoxamine phosphate had been prepared free from pyridoxal phosphate as indicated by the tyrosine decarboxylase apoenzyme, which responds only to pyridoxal phosphate, or to pyridoxal in the presence of ATP, but not to pyridoxamine phosphate or pyridoxamine. The glutamic-aspartic apotransaminase is activated by pyridoxal or pyridoxamine under proper conditions, but may be prepared in a state which responds only to the respective phosphates.

These data constitute biological evidence in support of the proposed mechanism of the function of pyridoxal phosphate as coenzyme of transamination. Thus pyridoxamine phosphate and pyridoxamine have been shown to be the equivalents of pyridoxal phosphate and pyridoxal, respectively, for transamination, though not for amino acid decarboxylation.

G24. Factors Influencing the Anaerobic Production of Gas by Bacillus subtilis.

NATHAN R. SMITH AND MARIE E. WENZEL, Plant Industry Station,
U. S. Department of Agriculture, Beltsville, Md.

The anaerobic production of gas by some strains of *Bacillus subtilis* has been used recently to divide the species into two sections: one, the so-called Marburg strain of *B. subtilis*; the other, the Ford strain of *B. subtilis* or *B. licheniformis*. Using recommended media containing glucose or nitrate, or both together, a survey was made of some 40 strains of *B. subtilis*, which were found to correspond to Ford's broad interpretation of the species. The results showed that certain strains would not produce gas anaerobically in any of the media tried. Others would usually produce gas when both glucose and nitrate were present. Gas from glucose or nitrate alone occurred less often and irregularly. The addition of metallic iron to certain media containing glucose made conditions more favorable for gas formation; in other media it was without effect. The same held true for yeast extract and for phosphate. Growth was sometimes greatly reduced, sometimes increased, by anaerobic conditions and bore no relation to the gas produced. Two strains from Ford formed gas from nitrate in only 50 per cent of the 12 trials on standard media; but with glucose also added, they nearly always formed it. Different batches of the same medium failed to give identical results. From a large number of observations, it is concluded that the anaerobic production of gas by *B. subtilis* is a variable character dependent upon a number of factors, some of which cannot be controlled.

G25. Rapid Identification of Certain Clostridia by Plate Cultures on Medium Containing Egg Yolk. L. S. McCLUNG AND RUTH TOABE, Indiana University, Bacteriological Laboratories, Bloomington, Ind.

Addition of sterile egg yolk suspension to 4 per cent proteose peptone no. 2

agar (with mineral salts and glucose) provides a medium for rapid identification of certain clostridia (Nagler or LV reaction). Colonies of *Clostridium perfringens* (*C. welchii*) and the *C. sordelli*-*C. bifementans* group are surrounded by a wide zone of precipitation. These species may be separated by the rapidity of sporulation and the inability to ferment lactose by the latter cultures. Colonies of *C. oedematiens* (*novyi*) type A are surrounded by a smaller, more intense precipitation area and a metallic luster extending from the edge of the colony outward but not to the edge of the precipitation zone. This luster is marked with radial striations. Type B colonies show the precipitate zone but no luster. The colonies of *C. parabotulinum*, types A and B, and those of type B *C. botulinum* are covered with a luster which extends in a regular circular zone beyond the colony edge and to the edge of the area of precipitation. Colonies of types C, D, and E of *C. botulinum* have the typical wide precipitate zone, but only a narrow luster band following the contour of the colony edge. *C. sporogenes* colonies are covered with luster and show precipitate under the colony, but neither extends beyond the colony edge. *C. tertium*, *C. tetani*, *C. histolyticum*, *C. septicum*, *C. capitovalis*, *C. chawoei*, and *C. cochlearium* show no reaction on this medium.

G26. *Relationships of Proshigella dispar to Other Proshigella and Shigella Species.* PHILIP L. CARPENTER, Rhode Island State College, Department of Bacteriology, Kingston, R. I.

More than 150 strains of *Proshigella dispar* and other *Proshigella* species isolated in this country and Europe have been studied. These organisms are so diverse physiologically that serological grouping is most feasible. Within a single serotype, biochemical properties vary widely. Antigenic analysis indicates a multiplicity of antigenic components, many of which are common to several types. The overlapping of physiological and serological properties re-emphasizes the spectrum nature of characteristics within this genus of the *Enterobacteriaceae*.

G27. *The Classification of Paracolon Bacilli Isolated from Man.* MACDONALD FULTON AND MARTHA L. CHILTON. University of Texas School of Medicine, Department of Pediatrics, Galveston, Texas.

Paracolon strains were isolated from feces or rectal swab specimens from 239 adults and 31 children, and, in addition, 22 miscellaneous sources. Some of the subjects were healthy, others had acute or chronic diarrhea. The IMVIC reactions and other customary bacteriologic tests were studied, together with fermentation tests in adonitol, aesculin, melezitose, and melibiose. Preliminary serologic studies indicated the possibility of recognizing many paracolon types promptly by the slide macroscopic agglutination test. Improved methods of demarking the group from *Salmonella* and *Proteus* were investigated.

G28. *A Study of Adonitol-fermenting Paracolon Bacilli.* MARTHA L. CHILTON AND MACDONALD FULTON. University of Texas School of Medicine, Department of Pediatrics, Galveston, Texas.

A collection of 50 paracolon strains which fermented adonitol was examined in detail. There was no bacteriologic or antigenic similarity to Rettger's bacillus. In IMVIC reactions 28 strains were ++-- (*Escherichia coli* type), 10 were --++ (*Aerobacter aerogenes* type), and 12 were intermediate in reactions. None of the strains hydrolyzed gelatin or produced sulfide. The limited distribution among bacteria of the ability to ferment this carbohydrate makes it a characteristic which appears to be of value in recognizing paracolon bacilli.

G29. *A Study of the Genus Microbacterium.* R. N. DOETSCH AND O. N. ALLEN, University of Maryland, Department of Bacteriology, College Park, Md.
Paper withdrawn before presentation.

G30. *Studies on Myxobacteria.* ERLING J. ORDAL, University of Washington, Department of Bacteriology, Seattle, Wash.

Studies have been made on fruiting body production, on the surface characteristics of vegetative cells, and on the virulence of a number of strains of *Chondrococcus columnaris*, a myxobacterium pathogenic to fish. It is concluded that the fruiting bodies occurring in dilute aqueous solutions may be considered as normal types. Fruiting bodies may be demonstrated by placing autoclaved fingerling fish in large flasks containing sterile tap water and inoculating with *Chondrococcus columnaris*. Fruiting body formation is normally evident after a few days. The fruiting bodies are usually columnar, sometimes highly branched structures extending a distance of 0.5 to 3 millimeters above the surface of the fish. They may be packed closely together covering the entire surface of the fish. Atypical fruiting bodies have been observed with old laboratory cultures, and with freshly isolated cultures of low virulence. Evidence of a relationship between virulence and surface characteristics of vegetative cells has been obtained.

G31. *Bacterial Variation, Population Dynamics, and Selective Environments.* WERNER BRAUN, University of California, College of Agriculture, Department of Veterinary Science, Berkeley, Calif.

Continued studies on dissociation in *Brucella abortus* have confirmed previous reports regarding the role of inherent and certain environmental factors governing population dynamics in controlling bacterial variation. This information permits better understanding of the apparent instability of bacterial mutants, the apparent ability of bacteria to adapt themselves to altered environmental conditions, and the occurrence of apparently orderly successive changes. It also supplies experimental evidence for certain general problems of evolution dealing with the relationship of size of the breeding population, mutation rates, and generation time to the accumulation of mutations within isolated populations.

Use of selective environments can alter the selection value of arising variants and prevent their establishment; thus, addition of as little as 2 per cent of normal serum of rabbits, cows, or hogs to broth cultures will usually prevent

dissociation from the S type. This is due to the presence in normal serum of factors suppressive to R and Br variants of *B. abortus*. If dissociation does occur, the variants represent types ordinarily not observed; presumably these types have little chance to establish themselves when in competition with R and Br. The suppression of dissociation is not due to the general bactericidal action of sera, since sera heated at 56 C for one hour will still suppress dissociation. Specifically selective environments, which will permit the establishment of one chosen variant only, may be produced through the addition of antiserum which contains antibodies for many variants but has been absorbed by the one desired variant.

G32. Induced Resistance of *Staphylococcus aureus* to Antibiotics. JOHN W. KLIMEK, CHESTER J. CAVALLITO, AND JOHN HAYS BAILEY, Winthrop Chemical Company, Inc., Division of Research, Rensselaer, N. Y.

Resistance of *Staphylococcus aureus* to various antibiotics *in vitro* has been studied by subculturing the organisms in the presence of progressively increasing amounts of antibiotic substance. The rate and degree of resistance acquired was found to vary with each antibiotic. Slight resistance, even after many transfers, was developed by cultures exposed to gliotoxin and the active principle of *Allium sativum*. No appreciable resistance was developed in the presence of aspergillic acid. Under similar conditions marked increase in resistance was developed to penicillin, streptomycin, pyocyanin, and the active principle of *Asarum reflexum*. In the case of streptomycin, development of resistance occurred most rapidly.

Increase in resistance to any one of the antibiotics in no way affected its sensitivity to any of the others, regardless of their nature or composition. Reversal to sensitive state was found to be rapid, regardless of time required to develop high or low degrees of resistance.

G33. The Development of Penicillin Resistance by *Meningococcus* *in Vivo*. C. PHILLIP MILLER AND MARJORIE BOHNEHOFF, The University of Chicago, Department of Medicine, Chicago, Ill.

In a preliminary communication, the authors reported the development of penicillin resistance *in vitro* by repeated subcultivation of meningococci on solid media containing increasing concentrations of penicillin. Continuation of those studies has raised the level of tolerance previously described; e.g., one strain of meningococcus has acquired the ability to grow on media containing 65 units of penicillin per ml. Penicillinase has not been demonstrated in any of the strains made penicillin-resistant *in vitro*.

Penicillin resistance was developed *in vivo* by the following method: mice were infected with varying numbers of meningococci suspended in mucin and treated with subcurative doses of penicillin. Cultures of heart blood made at autopsy on penicillin-free media from mice dying of the infection or killed at convenient intervals in its course were used to infect the next group of mice.

At the beginning of the series, 20–40 units of penicillin sufficed to cure mice infected with approximately 10^8 meningococci. After 25 passages through penicillin-treated mice this strain has developed sufficient resistance to produce infections which regularly proceed to fatal termination with positive blood cultures, in spite of treatment with 1,400 units of penicillin. Penicillinase has not been demonstrated in this strain.

G34. Development of Streptomycin Resistance of the Shigellae. MORTON KLEIN AND LEONARD J. KIMMELMAN, University of Pennsylvania, Department of Bacteriology, Philadelphia, Pa.

Eleven strains of *Shigella paradysenteriae* and one strain of *Shigella dysenteriae* have been tested for their susceptibility to streptomycin. In extract broth, pH 7.2, all of the strains were inhibited in the range of 2 to 7 units of streptomycin per ml. Ten strains were tested and each was found to become rapidly streptomycin-resistant. Following a series of 7 broth subcultures of 0.1 ml inocula in increasing concentrations of streptomycin, 7 of the strains grew in 1,000 units per ml of streptomycin, the highest concentration tested. The 3 remaining strains became resistant at a slower rate.

By testing 400 one-tenth-ml samples from the original susceptible culture of *S. dysenteriae* (0.1 ml of an 18-hour broth culture inhibited by 5 units of streptomycin) it was possible to isolate in approximately 1 per cent of the 0.1-ml samples, bacteria which grew directly in 1,000 units of streptomycin. Continuous selection of streptomycin-susceptible colonies revealed that the susceptible cultures consistently gave rise to a small percentage of naturally resistant variants. Unless one tested a relatively large portion of a culture, the few highly resistant forms in a "susceptible" culture would readily be missed.

The streptomycin-resistant strains showed no change in their susceptibility to penicillin or sulfonamides. A combination of streptomycin and sulfadiazine or streptomycin and penicillin was more effective than either agent alone.

G35. The Susceptibility of Penicillinase-producing Bacteria to Penicillin. AMEDEO BONDI, JR., AND CATHERINE COLLINS DIETZ, Temple University School of Medicine, Department of Bacteriology and Immunology, Philadelphia, Pa.

Penicillinase-producing bacteria vary considerably in their sensitivity to penicillin. In spite of their potential ability to produce large amounts of this enzyme which destroys penicillin, some of these organisms are quite sensitive to this antibiotic agent. A number of factors including inoculum size and media ingredients influence the sensitivity of these organisms. Of great importance is the rate of growth of the organism under study. The slower-growing organisms are more sensitive due to the slow rate at which the enzyme is produced. Growth of such organisms is inhibited before sufficient quantity of the enzyme accumulates to destroy the penicillin present.

There is some evidence that if a suitable agent were available which would poison this enzyme or inhibit its production the susceptibility of certain of these organisms to penicillin could be increased.

G36. *Immunologic Aspects of Protein Metabolism.* PAUL R. CANNON, University of Chicago, Department of Pathology, Chicago, Ill.

Evidence will be presented indicating the importance of protein nutrition in the retention of resistance to bacterial infection, and for the production of antibodies. The basic role of essential amino acids in the fabrication of tissue protein and antibody globulin will be particularly emphasized, and experiments will be described illustrating ways in which the problem of the immunologic relationships of diet to resistance may be studied.

G37. *Some Applications of Physical Methods to Problems of Bacteriology.* RALPH W. G. WYCKOFF, National Institute of Health, Bethesda 14, Md.

During the last years a group of physical techniques has been developing for the detection of particles of submicroscopic and macromolecular dimensions, and for the determination of their essential physical properties and the physico-chemical characteristics of their suspensions. These tools of a molecular biophysics, as illustrated by ultracentrifugation, electrophoresis, and electron microscopy, have thus far been chiefly used in preparing and characterizing purified viruses, but they are equally applicable to many problems of bacteriology. The most obvious of these deal with the fine structure of bacterial cells, with the gross morphology of the smallest microorganisms, with the nature and properties of the products of bacterial metabolism, and with the intimate mechanism of the immune reaction. This paper is a discussion of the kinds of bacteriological problems to which such a biophysical approach has already been made, of the types of results that have been attained, of some of the potentialities and limitations of existing physical tools, and of some indications of the way this type of research may be expected to develop in the immediate future. Especial attention will be given to illustrating, through lantern slides, certain recent results of the electron microscopic study of bacteria and bacterial products.

G38. *Electron Microscopy of Bacterium tularensis.* HENRY T. EIGELSBACH, ENS., H(S), USNR, LESLIE A. CHAMBERS, AND LEWIS L. CORIELL, CAPT., MC, AUS, Camp Detrick, Frederick, Md.

The morphology of *Bacterium tularensis* as determined with the electron microscope is in agreement with a recent systematic study of the morphology of this organism by means of vital staining techniques and dark-field examination.

In general, *B. tularensis* possesses multiple morphological units including large and small coccoid and bacillary, oval, minute, filamented, bean-shaped, dumb-

bell, bizarre, and so-called "involution" forms. The suggestion of the existence of minute morphological units of less than 300μ in diameter was confirmed. The typical cell possesses little opacity to the electron beam and presents a semi-transparent, nebulous appearance. Critical examination for the presence of a cell wall revealed an extremely delicate structure of very low electronic density, which possibly accounts for the low survival rate when subjected to sonic vibration or the lyophilization process.

G39. The Structure of Spirochaeta novyi as Revealed by the Electron Microscope.

RUTH LOFGREN AND MALCOLM H. SOULE, University of Michigan, Department of Bacteriology, Ann Arbor, Mich.

Our early observations on the spirochetes of relapsing fever with the electron microscope revealed marked differences in structure depending upon the methods of preparing the specimens. Since pure cultures of these organisms are not available, it is necessary to resort to the use of infected blood as a source of material. The removal of the blood constituents by washing and repeated centrifugation was unsatisfactory and necessitated the development of a simpler procedure, namely, electrodialysis and suspension in distilled water. Organisms prepared in this manner possessed a uniform outline, pointed tips less dense than the cell contents, and granular or mottled protoplasm. A few of the forms had a long, rather heavy terminal filament extending from one end.

Following electrodialysis, suspensions of *Spirochaeta novyi* were allowed to stand 48 hr in distilled water. Examination of these cells revealed no apparent structural changes. The addition of distilled water with subsequent centrifugation and resuspension was repeated four times, with examination of the spirochetes after each washing. There was a progressive destruction of the cells; fragmentation of the periplast resulted in the production of numerous flagellalike fibers along the bodies of most of the organisms; and areas of pronounced swelling and granulation of the protoplasm were common. Large, dense granules, similar to those detected in frozen preparations of this organism, were frequently observed. It appears possible that the flagella attached to spirochetes observed by other investigators may be formed by the mechanical shredding of the cell wall as a result of the treatment of the specimens.

G40. Electron Microscope Studies of Organisms of the Pleuropneumonia Group.

WILLIAM E. SMITH, JAMES HILLIER, AND STUART MUDD, Rockefeller Institute, New York, N. Y.; RCA Laboratories, Radio Corporation of America, Princeton, N. J.; and University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The diversity of morphological forms exhibited by organisms of the pleuropneumonia group has led to the conception that these organisms are essentially different from the bacteria. Spherical bodies are prominent in all strains, and branched structures and filamentous forms have also been described, as well as

small rod-shaped bodies. The electron microscope was employed to gain further knowledge of these forms, and micrographs were made of a strain of organisms of the pleuropneumonia group isolated from the human cervix. This strain, L 50, had been accepted as a member of the group by all investigators who examined it. The electron micrographs show spherical forms and short filaments, and, more important, many small rod-shaped forms are present which have a well-defined bacillary structure with cell wall, cytoplasm, and various intracellular details. The findings indicate that the strain studied is essentially a highly pleomorphic bacillus. This is in accord with the view of Dienes concerning the nature of organisms of the pleuropneumonia group.

G41. *A Comparison of Electron Micrographs with Photomicrographs of Young Bacterial Cultures Stained to Demonstrate Desoxyribonucleic Acid.* RUTH A. C. FOSTER AND LELAND L. ANTES, University of Texas, Department of Bacteriology and Bureau of Engineering Research, Austin, Texas.

Young cultures of *Escherichia*, *Salmonella*, *Proteus*, *Serratia*, and *Corynebacterium* species, grown on solid medium and photographed with the electron microscope, show a spiral structure, opaque to electrons. This spiral is most convincingly demonstrated during the early logarithmic phase of bacterial growth when the cells are of maximum size. When these photographs are compared with light microscope photographs of cells of comparable age, stained with Wright's stain following osmic acid fixation and acid hydrolysis, the dark bodies variously called nucleoids, chromatinic bodies, or dumbbell-shaped bodies appear within the cells in positions which suggest that they are analogous to the spiral structure observed with the electron microscope.

G42. *Gram-positive Characteristics of the Neisseria.* JAMES W. BARTHOLOMEW, University of Southern California, Department of Bacteriology, Los Angeles 7, Calif.

Henry and Stacy (1943) and Bartholomew and Umbreit (1944) showed that gram-positive cells could be made to stain either gram-negative or gram-positive by removing or replating magnesium ribonucleate onto the cell. True gram-negative cells could not be rendered gram-positive in this manner. This was the principal approach for this investigation, although resistance to digestive agents and dye sensitivity were included.

Suspensions of *Neisseria catarrhalis*, *Escherichia coli*, and *Serratia marcescens* were prepared, and 0.1 per cent formaldehyde and 1 per cent magnesium ribonucleate were added. Slides were prepared at intervals and gram-stained. Suspensions of *N. catarrhalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *S. marcescens*, and *E. coli* were digested with 1 per cent solutions of trypsin, pepsin, and potassium hydroxide. Dye sensitivity was determined by presence or absence of growth on nutrient agar containing .001 per cent gentian violet.

N. catarrhalis accepted the magnesium ribonucleate and stained gram-positive after fifteen minutes' exposure. *E. coli* and *S. marcescens* remained gram-negative. *N. catarrhalis* was more resistant to the digestive agents than *E. coli* or *S. marcescens*, but was less resistant than *B. subtilis* or *S. aureus*. *N. catarrhalis*, *B. subtilis*, and *S. aureus* did not grow on the gentian violet agar; *E. coli* and *S. marcescens* did. Hence, *N. catarrhalis* is structurally and physiologically related to gram-positive bacteria.

The implications of these results on the division of bacteria into gram-positive and gram-negative groups will be presented later.

G43. *Reproductive Processes in Proteus Cultures.* L. DIENES, Massachusetts General Hospital, Department of Pathology and Bacteriology, Boston, Mass.

Proteus starts to grow in the first few hours in short bacillary forms without tendency to spread. At a certain moment the bacteria grow into filaments, are provided abundantly with flagella, and begin to swarm on the surface of the agar. In nearly all strains a few bacilli develop large round bodies attached to their sides. The swarming filaments of such strains transferred to tap water show bacteriopsis (the contents of the filaments are extruded in the form of droplets attached to the filament). Some of these enlarge within a few minutes to a large round body. This is not a purely physical phenomenon because the round bodies have a resistant membrane and germinate in transplants. Like the round bodies in other species, they usually produce a tiny "L"-type colony; rarely they fractionate and reproduce bacteria of the usual morphology. When the spreading filaments of two appropriate strains meet on the surface of the agar, in the area of juncture the majority of the long filaments are transformed quickly into large bodies. These increase in size until they fractionate and reproduce typical bacterial forms. "L"-type colonies, in contrast to the large bodies produced in tap water, are rarely formed. These phenomena are produced only when spreading filaments meet spreading filaments; meeting colonies of other strains growing as small bacillary forms does not produce this effect. Such observations suggest a sexual process but a union either between bacterial filaments or between large bodies was not observed.

G44. *The Size of Living Bacteria, Measured with the Phase Microscope.* OSCAR W. RICHARDS, American Optical Co., Scientific Instrument Division, Research Division, Buffalo 11, N. Y.

Bacteria living in a suitable culture medium are revealed clearly without staining with the Spencer phase microscope, either brighter or darker than their environment. The diffraction patterns which obscure unstained bacteria observed with bright-field microscopy are avoided. The phase microscope uses optical path differences introduced into its lenses to make visible regions of different refractive index in the specimen. By the use of an intense light source

the photographic exposures can be made short enough to avoid blurring from brownian movement. Measurements of *Bacillus megatherium* will illustrate the method.

G45. Nuclear Staining of Escherichia coli. JEAN LOVE PALMER, Carnegie Institution of Washington, Department of Genetics, Cold Spring Harbor, N. Y.

The Robinow technique for staining bacterial nuclei was modified to yield reproducible results with *Escherichia coli* (strain B). The length of each step in the procedure (fixation in OsO₄ vapor, hydrolysis in HCl, neutralization in buffer, staining in Giemsa solution) must be carefully chosen, for best results, with organisms from both liquid and solid media.

Changes in nuclear morphology during the growth of a culture were studied. Cells from fully grown cultures appear lightly but uniformly stained; nuclear differentiation begins to appear soon after transfer to a fresh medium while cell elongation without division takes place. In actively growing cells, the nuclei seem to divide before the bacterial cell itself does, so that the common longer cell forms show at least two nuclei, while the small, recently divided individuals contain one nucleus only.

The nuclear structures of abnormal cells produced by various methods have also been examined.

A1. Is Rinse Water at 170 F or Higher Essential to Produce "Sterile" Eating and Drinking Utensils? MURRAY P. HORWOOD, Massachusetts Institute of Technology, Department of Civil and Sanitary Engineering, Cambridge 39, Mass.

The Massachusetts Institute of Technology Graduate House dining service has been under technical supervision since 1943. Eating and drinking utensils are soaked and thoroughly desoiled in clean soapy water at 110-120 F, and passed through a mechanical dishwashing machine for washing and rinsing. The temperature of the water varies between 160-180 F and usually is at 170 F. The total time in the dishwasher is 35 seconds. All utensils are allowed to dry in the air. Bacteriological results have been consistently satisfactory and approximated sterility. In the course of the work it became obvious that the excellent bacteriological results were due to the effective preliminary desoiling of all utensils. Since the temperature of the wash and rinse water could be regulated, a comparative study, using rinse water at 145-150 F and rinse water at 160-180 F, was undertaken. All other factors were kept constant. Five test runs over weekly intervals clearly demonstrated that "sterile" utensils can be obtained with rinse water at 145-150 F, as with rinse water at 160-180 F, if the preliminary desoiling is complete and effective. The emphasis in restaurant sanitation should therefore be on adequate preliminary desoiling of all utensils rather than on the use of high temperature rinse waters.

A2. The Viability of Dried Lactobacillus bulgaricus Cultures as Affected by the Temperature of the Reconstituting Fluid. MARVIN L. SPECK AND ROBERT P. MYERS, Sealtest, Inc., Research Laboratories, Baltimore, Md.

Relatively little attention has usually been directed to the temperature of the reconstituting fluid when plating readily soluble dried milk products. However, the use of warm water (35–55 C) has been employed by a number of workers to improve the dispersion of the dried products. The procedure we adopted is to add water at 50 C to the powder which has been weighed into a wide-mouthed jar or bottle, shake the mixture vigorously fifty times, let stand five minutes (in a bath) at 50 C, again shake vigorously fifty times, then dilute in water blanks at room temperature. We have found that the number of viable cells in spray-dried skim milk cultures of *Lactobacillus bulgaricus* are greatly increased if the temperature of the reconstituting fluid is 50 C rather than 21–25 C. It is essential that the reconstituting liquid be at 50 C when added to the powder rather than that the mixture be warmed to 50 C from room temperature after the liquid is added.

Reconstitution at 50 C instead of at room temperature shows a proportionately greater increase in viable cells with older cultures than with freshly dried ones. This heat treatment has not resulted in an increase in the count of viable cells in a dried culture of lactic streptococci, but rather a decrease. The counts on commercial spray and roller-dried milk powders has at times given increased counts and at others decreased counts by reconstitution in water at 50 C.

A3. A Method for the Production of Antiserum Specific for Lancefield Group D Streptococci. IRENE J. UHRIK AND J. J. REID, The Pennsylvania State College, Department of Bacteriology, State College, Pa.

Although the Lancefield method for the production of streptococcal grouping serum usually yields excellent antiserum for groups A, B, and C, considerable difficulty has been encountered by many investigators in attempts to produce antiserum for group D by this method. Following numerous attempts, a method has been devised by the authors which has produced good serum in most instances. Cells of 18-hour cultures of Lancefield strain C-1 grown in 100 ml of glucose veal infusion broth are removed by centrifugation and extracted at room temperature for 30 min in 50 ml of acetone. Extraction is twice repeated, following which the centrifuged cells are dried *in vacuo* over H_2SO_4 at 10 C for 24 hours. The dried cells are then suspended in 0.85 per cent saline containing 0.2 per cent formalin and held at 10 C for 48 hours. This stock antigen is then diluted 1 to 20 with saline solution and used in the rapid immunization of rabbits. Following an initial dose of 0.1 ml, dosage is rapidly increased to a maximum of 1.0 ml daily and injections are continued until a total of 40 ml have been administered. Injections are continuous unless the physical condition of the animal warrants an occasional brief period of rest. Antiserum produced in this manner is used with freshly prepared testing antigen.

A4. Isolation of Enterococci from Natural Sources. CHARLES E. WINTER AND LESLIE A. SANDHOLZER, Fish and Wildlife Service, U. S. Department of the Interior, and the University of Maryland, College Park, Md.

Using a modification of the White and Sherman sodium azide penicillin medium for isolation of the enterococci, studies have been made to determine the possibility of using these microorganisms as an index of fecal pollution. The medium was modified by doubling the penicillin content and adding 0.001 per cent methylene blue. Isolates from the medium were verified by the following tests: catalase production, salt tolerance, growth at 10 C and 45 C, and reaction on litmus milk. Only those gram-positive streptococci which met these requirements, as suggested by Sherman's studies, were considered to be typical enterococci.

Enterococci were isolated from raw sewage, fresh and salt water. When compared with the numbers of coliform bacteria present in the same samples, the enterococci are usually present in much smaller quantities. In polluted waters, coliform bacteria persisted for a greater distance from the source of pollution than did the enterococci. Studies are now in progress to determine the survival period in sewage and water and the seasonal quantitative variations which might occur.

Feces of man and certain domestic and wild animals were examined quantitatively for the presence of enterococci. Fecal samples of the latter were taken from animals which had been trapped in their natural environment. Although the numbers of coliform bacteria per gram of fresh feces were roughly constant, there was marked variation in the enterococci count. The numbers of enterococci per gram varied from zero in the muskrat to a maximum of 37,000,000 in the raccoon.

A5. Further Studies of a More Rapid Method for Detecting Coliform Bacteria in Natural Waters and Shellfish. FREEMAN H. QUIMBY, LESLIE A. SANDHOLZER, JOHN COX, AND CLIFFORD BYRD, Washington Missionary College, U. S. Fish and Wildlife Service, and Crisfield Seafoods Laboratory, Takoma Park, Md., and College Park, Md.

Use of a sodium lauryl sulfate tryptose nitrate broth reduces the time required for the presumptive test for coliform bacteria in water to twelve hours or less. Employing the principle of nitrate reduction instead of lactose fermentation, a new, more rapid, and possibly more reliable basis for the presumptive test was introduced.

Since the publication of the results of the test on raw and treated waters, the investigations have been extended to include sea water, crabmeat, and oysters. The results of the test applied to twelve samples of oysters, forty-eight samples of crabmeat, and fifty samples of sea water were comparable to those obtained by the standard method for determining coliform bacteria.

The data obtained thus far indicate that it is not necessary to confirm tests which are nitrite-positive within eight hours or less. Since the majority of samples tested gave positive tests for nitrites between six and eight hours, it is clear that the method may be four to eight times more rapid than the standard procedure.

Sixty additional samples of water have been tested using the medium as a secondary medium for confirmation after preliminary growth in lactose broth. Eighty-eight per cent of the samples that were gas-positive in lactose broth produced nitrites in four hours or less. Ten hours was the longest incubation required of the remaining samples to produce nitrites. All samples that were nitrite-positive were also positive in the standard confirmatory media.

A6. The Cellulose-decomposing Bacteria in the Rumen of Cattle. R. E. HUNGATE, State College of Washington, Department of Bacteriology and Public Health, Pullman, Wash.

A method for growing the cellulose-digesting bacteria in the rumen of cattle has been developed. Quantitative estimates of the number of these bacteria have been made and their numbers found to range from 20,000 to 1,000,000,000 per ml. Cellulose decomposition is very active, clear spots developing in the agar within three days. Two chief types of bacteria have been pure-cultured. One is a gram-negative streptococcus and the other a rod form. Slight amounts of gas may be produced but in general the metabolism results in the production of acid without gas. It is believed that the isolated organisms are responsible for most of the cellulose decomposition which takes place in the rumen.

A7. A Survey of the Coliform Status and Suggested Standards for Coliform Control of Pasteurized Milk in a Large City. LEON BUCHBINDER AND JOHN W. FERTIG, Department of Health, Bureaus of Laboratories and Food and Drugs; Columbia University, School of Public Health of the Faculty of Medicine, New York, N. Y.

In the absence of standards for coliform control of pasteurized milk other than those based almost solely on empirical grounds a survey of the coliform status of pasteurized milk in New York City was conducted for a one-year period so that reasonable standards might be established. Quart samples from each of forty-six pasteurization plants were studied once weekly. Three volumes from each sample were examined simultaneously: 1 ml, 20 ml, and the remainder of the quart. A total of about 2,150 samples were studied on desoxycholate agar. It was found that the percentage of samples positive for coliforms varied markedly with season. The July peaks for the three volumes in descending order are 86 per cent, 58 per cent, and 24 per cent, whereas the December lows are 59 per cent, 25 per cent, and 3 per cent, respectively. The 20-ml volume data were used to suggest practical standards. The numbers of coliform organisms per ml which were exceeded by 25 per cent, 20 per cent, and 10 per cent, respectively, of the samples for each four-week period were examined. It was found that the critical numbers for the several percentages in order were 0.1, 0.1, and 0.5 in the six cool months and 0.5, 1, and 2 in the six warm months. Comparison of the findings for summer and winter in individual plants suggests the existence of a definite pattern. Trial standards of 0.1 with a zone of grace to 0.3 for the cool months and 1 with a zone of grace to 2 for the warm months were established. Tables have been prepared which indicate the volume of samples required to differentiate

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between the upper and lower limits of the zones with a 90 per cent probability of accuracy.

A8. The Effects of Quality and Pasteurization of Milk on the Bacterial Flora and Quality of Cheddar Cheese. RALPH P. TITSLER, DONNA S. GEIB, GEORGE P. SANDERS, HOMER E. WALTER, OSCAR S. SAGER, AND HARRY R. LOCHRY, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington 25, D. C.

Cheddar cheese was made from good, fair, poor, and very poor milks. One half of each lot of milk was pasteurized. The bacterial flora of each cheese was determined at 1 day, 2 weeks, and 1, 2, 3, 4, and 6 months. The bacterial counts of raw milk cheese were from 2- to 1,000-fold greater than those of the corresponding pasteurized milk cheese, depending on age of cheese and quality of milk.

During the first month of curing, the counts of raw milk cheese decreased approximately 10-fold. Subsequently, depending on quality of milk, the counts increased greatly or remained relatively constant. During the first month, the counts of pasteurized milk cheese decreased from 50- to 100-fold and subsequently remained relatively constant or decreased slightly. Cheese made from poor and from very poor milk, raw or pasteurized, contained many more bacteria than did cheese from good and from fair milk.

Cocci from the lactic starter predominated in young cheese, regardless of quality or pasteurization of milk. After one month, the flora of pasteurized milk cheese consisted almost entirely of enterococci, and that of raw milk cheese of lactobacilli, enterococci, and a few diversified types.

The bacteriological quality of the milk, both raw and pasteurized, was an important factor affecting the quality of the cheese. With high-grade milk there was very little difference in quality between the raw milk and the pasteurized milk cheese, but with low-grade milk the raw milk cheese was inferior.

A9. A Survey of Antibiotic Production by Representative Aspergilli, Penicillia, and other Fungi from a Culture Collection. ALBERT KELNER, University of Pennsylvania, School of Medicine, Philadelphia 4, Pa.

Over 100 species and strains of *Aspergillus* and *Penicillium*, as well as some members of the genera *Gliocladium*, *Trichoderma*, *Scopulariopsis*, *Metarrhizium*, and *Paecilomyces* were studied for antibiotic production. The cultures were selected by Dr. Charles Thom as representative of the groups and subgroups of the genera. Most of them came from the collection of the Northern Regional Research Laboratory, Peoria, Illinois. Each mold was grown in surface culture in at least four media, glucose peptone yeast extract broth, corn steep lactose broth, Czapek-Dox and Raulin-Thom broths. The crude filtrates were tested for activity against *Staphylococcus aureus*, *Escherichia coli*, *Eberthella typhosa*, and *Pseudomonas aeruginosa*.

The problems encountered in making the survey will be presented. The results obtained will be discussed with special reference to the question of

whether antibiotic production is a characteristic of a particular strain or the species as a whole.

A10. *Eumycin*—a New Antibiotic Active Against Pathogenic Fungi and Higher Bacteria, Including Bacilli of Tuberculosis and Diphtheria. EDWIN A. JOHNSON AND KENNETH L. BURDON, Baylor University College of Medicine, Department of Bacteriology and Immunology, Houston, Texas.

By exposing agar plates to dust and isolating bacterial colonies inhibiting growth of neighboring mold colonies, strains of *Bacillus subtilis* were obtained from which we have extracted a new antibiotic ("eumycin") active against filamentous pathogenic fungi and higher bacteria. To date maximum yield has been obtained from cultures five days old at 30 C in buffered yeast extract proteose peptone broth. The substance is soluble in butyl alcohol, ethyl alcohol, and acetone, but not in ether or amyl acetate. By precipitation from the original broth with acid, extraction of the precipitate with alcohol, evaporation of the alcohol, and re-solution with dilute NaOH to pH 7.0, the active material is greatly concentrated. The final (Berkefeld-filtered) solution is nearly colorless, heat-stable in acid, unstable in alkaline solutions beyond pH 8.0. It has low toxicity for mice. Eumycin has no action on typhoid or colon bacilli, and only slightly inhibits staphylococci. In concentrations of 0.1–0.3 mg (dry weight) per ml of medium it prevents entirely, or definitely inhibits, the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Epidermophyton floccosum*, and related species of fungi, *Actinomyces*, and *Mycobacterium tuberculosis* (avian and human types). Its effect on species of *Sporotrichum* and *Hormodendrum* is slightly less, and it fails to inhibit *Monilia* or *Cryptococcus*. Greatest bacteriostatic activity is exhibited against *Corynebacterium diphtheriae*, for as little as 0.005 mg per ml stops growth. Studies of possible therapeutic value in experimental infections are in progress.

A11. Two Antibiotics Produced by *Actinomyces* Isolated from Soil. ALBERT KELNER, WALTER KOCHOLATY, RENATE JUNOWICZ-KOCHOLATY, AND HARRY E. MORTON, University of Pennsylvania, School of Medicine, Departments of Physiological Chemistry and Bacteriology, Philadelphia 4, Pa.

Two antibiotics active not only against gram-negative but also many gram-positive organisms have been obtained from *Actinomyces* isolated from soil. Chemical studies thus far have made it possible to differentiate the two antibiotics from one another and from other known antibiotics. One antibiotic is produced by an actinomyces, A-10, which has been identified tentatively as belonging to the *A. lavendulae* group; the other antibiotic is produced by a strain, A-105, tentatively identified as a variant of *A. erythreus* or a new species. Cultural conditions for maximum yields of the antibiotics will be discussed. Chemical purification has resulted in a 40–50-fold increase in activity calculated on a

dry-weight basis. The antibacterial spectrum, toxicity for mice, and other aspects of the antibiotics will be discussed.

A12. *The Effect of Subtilin in the Treatment of Experimental Infections in Animals.*

A. J. SALLE AND GREGORY J. JANN, University of California, Department of Bacteriology, Los Angeles, Calif.

Subtilin, an antibiotic extracted from *Bacillus subtilis*, has been shown to be antagonistic chiefly against gram-positive bacteria. Acid-fast organisms, including *Mycobacterium tuberculosis* and a number of pathogenic higher fungi, are also susceptible. The agent is bacteriostatic in high dilutions and germicidal in greater concentrations.

Subtilin is a polypeptid, and is digested by proteolytic enzymes, such as pepsin, trypsin, and pancreatin. It shows its greatest activity at pH 2.2 and becomes slowly inactivated with decreasing acidity. At pH 7.0 it retains 94 per cent of its original activity; at pH 8.0 about 80 per cent; and at pH 9.0 about 65 per cent. Subtilin is relatively nontoxic when tested by the tissue culture technique. It is approximately 20 times more toxic to *Staphylococcus aureus* than to chick heart tissue, a remarkably low toxicity. A unit of subtilin has been determined and is defined as that amount present in 1 ml of the highest dilution (expressed in mg) capable of killing *S. aureus* in 10 min at 37 C (FDA phenol coefficient method).

Subtilin exhibited great activity *in vivo*. White mice were infected with a virulent culture of pneumococcus type III and treated after varying periods. Control animals died within 24 hr; all treated animals survived after receiving very minute amounts of the antibiotic. Guinea pigs infected with *B. anthracis* recovered after treatment with subtilin; control animals died within 36 hours. The antibiotic has shown a definite suppressive effect on the course of experimental tuberculosis in guinea pigs.

A13. *Reversal of Antibacterial Action.* JOHN HAYS BAILEY AND CHESTER J.

CAVALLITO, Winthrop Chemical Co., Inc., Research Laboratories, Rensselaer, N. Y.

Certain antibacterial agents are capable of being inactivated by cysteine. This report presents evidence that the bacteriostatic action of certain antibacterial agents may be reversed by cysteine.

By use of the Warburg technique it is possible to show with certain antibacterial agents that the cessation of oxygen uptake by the test organisms may be resumed upon the addition of cysteine to the bacteriostatic system. Another group of antibacterial agents, including penicillin and the active principle of *Asarum reflexum*, do not permit this reversal of bacteriostasis following treatment with cysteine.

A14. *The Action of Clavacin, a Clavacin Isomere, and Related Compounds on*

Tetanus Toxin. BRUNO PUETZER AND THOMAS C. GRUBB, Research Laboratories, Vick Chemical Company, Flushing, N. Y.

The synthesis of a clavacin isomere and related compounds was recently reported from these laboratories. It was believed of interest to determine the action of these compounds on tetanus toxin since Neter showed that clavacin neutralized this toxin *in vitro*. The clavacin, prepared according to Raistrick's method, isoclavacin, dimethylisoclavacin, and α -keto- $\beta(\beta$, β -dimethylacryl)-butyrolactone were mixed with 1:50,000 dilution of tetanus toxin in nutrient broth (pH 7.0), incubated one hour at 37 C, and injected subcutaneously into the hind legs of mice. Clavacin prevented tetanus and death of all mice, thus confirming Neter's report. Neither isoclavacin nor the two related compounds prevented tetanus or prolonged the lives of the animals beyond that of the toxin controls which died within 96-110 hours. Clavacin differs structurally from isoclavacin only in the position of one double bond. However, this simple shift in the position of the double bond produces a profound change in several of the chemical characteristics of clavacin. While the tetanus toxin-neutralizing activity of clavacin is apparently a function of the position of the double bond, which chemical group or groups in the molecule affected by the position of the double bond are responsible for the toxin-neutralizing properties cannot be postulated from the present evidence.

A15. The Inhibitory Action of Saliva on the Diphtheria Bacillus. The Antibiotic Effect of Salivary Streptococci. RICHARD THOMPSON AND MADOKA SHIBUYA, University of Colorado School of Medicine, Department of Bacteriology, Denver, Col.

This report is concerned with the inhibitory action of saliva on *Corynebacterium diphtheriae*; the role of salivary streptococci in this inhibition; and several factors which influence it. The method used involved placing standard drops of the material to be tested on pour plates containing suitable dilutions of bacilli and observing the zones of inhibited growth around the drops.

Pure cultures of "mitis" type of the viridans streptococci isolated from saliva inhibited the growth of the bacilli in the same manner as did fresh saliva. Removal of the streptococci from saliva by centrifugation, heat, or the bactericidal effect of copper abolished the inhibitory power. The actions of saliva and of pure cultures of streptococci were affected in identical fashion by several factors. Both actions were best demonstrated when the tryptose content of the medium was between 0.2 per cent and 0.5 per cent, and were completely eliminated on medium containing 2 per cent tryptose. Both actions were antagonized by certain organisms present in saliva, especially staphylococci. The actions of both saliva and cultures of streptococci were increased by their suspension in nutrient media rather than in saline. The increased activity was associated with increased growth of the streptococci. The inhibitory action of saliva was destroyed by 56 C in approximately the same time as was the action of pure cultures of streptococci. The destruction paralleled the diminution of the numbers of streptococci.

It is concluded that the inhibitory action of saliva against *C. diphtheriae* demonstrated by the method used is due to inhibitory streptococci present in the saliva.

- A16. *Changes in the Bacterial Flora of the Throat and Intestinal Tract During Prolonged Oral Administration of Penicillin.*** MIRIAM OLMSTEAD LIPMAN, JAMES A. COSS, AND RALPH H. BOOTS, Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital and the Columbia University, College of Physicians and Surgeons, Department of Medicine, New York, N. Y.

A bacteriological study on the throat and intestinal flora of ten rheumatoid arthritis cases, prior to and during the oral administration of half a million to a million units of penicillin daily over a period of months, has demonstrated a rapid and striking change. The prepenicillin throat cultures of all cases except one, which harbored Friedländer's bacillus, have shown the predominance of gram-positive diplococci, sensitive to penicillin. In the majority of cultures taken during the course of penicillin therapy, gram-negative organisms, resistant to penicillin, have predominated. Coliform bacteria, in no instance found prior to penicillin therapy, have appeared in most of the cultures during therapy. Changes in the intestinal flora, though less striking, have been definite. Non-hemolytic streptococci, recovered from most of the stool specimens before penicillin, have been found only infrequently during treatment. The relation of the concentration of penicillin in the serum to the sensitivity of the organisms isolated has been determined.

- A17. *Effect of Natural and Synthetic Rubbers upon the Stability of Penicillin and Streptomycin Solutions.*** J. B. HUELSEBUSCH, I. W. GIBBY, AND M. J. FOTER, The Wm. S. Merrell Co., Department of Bacteriology, Cincinnati 15, Ohio.

Following Cowan's report that synthetic rubber inactivated penicillin solutions, we have studied the effects of a number of synthetic and natural rubbers upon the stability of penicillin and streptomycin. The antibiotic solutions were placed in suitable lengths of sterile rubber tubing and allowed to stand at room temperature. At appropriate intervals, samples were withdrawn for assay. Twelve samples of synthetic rubber and four samples of natural rubber were tested. Of these, four samples of synthetic rubber and one sample of natural rubber inactivated penicillin completely in 24 hours. Two other samples of synthetic rubber caused marked reductions in the activity of penicillin during the same period. In six hours buna s rubber caused a 50 per cent reduction, and two other samples of synthetic rubber caused 20 and 30 per cent reductions in the activity of penicillin.

None of the samples of rubber tested caused any reduction in the activity of streptomycin.

- A18. *Submerged Culture of Molds for Amylase Production.*** B. BALANCHURA, F. D. STEWART, R. E. SCALF, AND L. A. SMITH, Joseph E. Seagram and Sons, Inc., Research Department, Louisville, Ky.

Barley malt possesses two disadvantages as a conversion agent for grain mashes. These are the high bacteria count and the excessive time required

for conversion of limit dextrins. Previous work on the use of mold amylase preparations revealed that they convert limit dextrins more rapidly than barley malt and result in higher yields of alcohol. Production of mold amylases by the submerged culture process would eliminate the problem of bacterial contamination.

Twenty-three strains of the genera *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* known to produce amylases were subjected to screening tests to determine their amylase production in submerged culture. The effect of pH, aeration, medium, and incubation time was studied to ascertain the conditions necessary for maximum amylase elaboration. The best preparations as determined by amylase content were further evaluated by the yeast fermentation of grain mashes using these preparations as conversion agents.

It was found that the most critical factors were pH, medium composition, and time of incubation. The best of the cultures tested is an isolate believed to be a variety of *Aspergillus flavus*. Forty-eight hours' incubation of this culture in a medium consisting of 3 per cent distillers' dried solubles at an initial pH of 6.0 results in a preparation with very high diastatic activity. This preparation has been successfully used as a replacement for barley malt in the conversion of grain mashes.

A19. The Production of Fungal Amylases in Submerged Culture and Their Use in the Production of Industrial Alcohol. J. M. VAN LANEN AND E. H. LEMENSE, Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill.

Studies were made to develop submerged culture methods for preparing fungal amylases. The object was to devise procedures adaptable to large-scale production, especially in connection with grain alcohol distillery operations.

A large number of molds, therefore, were cultivated under aeration in a medium composed of thin stillage and corn meal. Enzyme production was determined both by dextrinization of starch and by the ability of the culture liquors to replace barley malt as the saccharifying agent in the alcoholic fermentation of grains.

Of over 350 cultures tested, only seven produced practical concentrations of alpha amylase under the conditions employed. Of these, *Aspergillus niger* NRRL 337 was superior, both in producing alpha amylase and in replacing barley malt. The medium finally adopted consisted of thin stillage supplemented with 1 per cent corn meal and 0.5 per cent calcium carbonate. On this medium *A. niger* NRRL 337 gave amylase potencies equivalent to 600-800 alpha amylase saccharification units per ml of mycelium-free culture liquor in 72 hours. Since these culture liquors contained about 3 per cent solids, on the dry substance basis they were approximately 12 times as potent in dextrinizing power as barley malt. In the alcoholic fermentation of corn, use of culture liquors at the rate of 10 to 15 per cent of the mash volume resulted in alcohol yields of 5.2 to 5.4 proof gallons per bushel as compared to 5.0 to 5.2 obtained with commercial barley malt.

- A20. *Bacteriophage in the 2,3-Butylene Glycol Fermentation.*** C. J. WILDER AND W. H. STARK, Joseph E. Seagram and Sons, Inc., Research Department, Louisville, Ky.

A filtration technique has been developed for recovering and maintaining a bacteriophage culture isolated from *Aerobacter aerogenes* fermentations of acid-hydrolyzed corn mash in a 2,3-butylene glycol pilot plant. This method was developed after noting that there was a great loss in bacteriophage titer upon using a Seitz filter for recovery. As a filter pad in the Seitz filter carried a negative charge, it was believed that the phage particles were being adsorbed from the solution. This was based on the assumption that the phage particles were charged positively. Upon the addition of CaCO_3 to the solution the titer of the phage was not lost during filtration and this procedure enabled a stock phage culture to be maintained in the laboratory. It is believed that the addition of CaCO_3 to the solution changed the charge on the Seitz filter pad from negative to positive, allowing the phage particles to pass through.

The bacteriophage cultures have been classified in respect to the sensitivity and immunity of strains of bacteria to the phage. Stock phage cultures are being carried in the laboratory and bacterial cultures have been immunized against them.

- A21. *Studies on the Nutritional Requirements of Actinomyces griseus for the Formation of Streptomycin.*** GEOFFREY RAKE AND RICHARD DONOVICK, Squibb Institute for Medical Research, Division of Microbiology, New Brunswick, N. J.

Beef extract, or corn steep liquor, is shown to be unnecessary for the formation of streptomycin by *Actinomyces griseus* grown in a medium consisting of soybean meal, glucose, sodium chloride, and water. The absence from a soybean medium of an inorganic salt, such as sodium chloride, leads to very little streptomycin formation and it is suggested that beef extract may supply some of the necessary salts. Preliminary studies indicate that sulfate ion may be substituted for chloride ion but that magnesium does not satisfactorily replace the sodium ion. In shake-flask cultures the volume of medium per flask significantly affects the yields of streptomycin obtained.

- A22. *The Influence of the Rate of Aeration on Oxidation-Reduction Potentials and Streptomycin Production by Actinomyces griseus.*** J. E. KEMPF AND P. SAYLES, University of Michigan, Hygienic Laboratory, Ann Arbor, Mich.

Because of variations in streptomycin titers under controlled aeration rates, it seemed desirable to determine the influence of this factor on oxidation-reduction (OR) values and streptomycin production.

Sterile beef extract broth, 350 ml, and sterile 50 per cent glucose, 7.5 ml, were placed in each special glass fermentation flask. The flasks and contents were then incubated at 28 C, and sterile air was introduced at rates varying between 0.5 and 6 volumes per volume of media per minute (vpm). Three groups of

experiments were carried out: sterile media control, media inoculated with *Actinomyces griseus*, inoculated media containing 0.1 per cent ferro-ferricyanide. The ferro-ferricyanide was added to obtain closer agreement of duplicate electrode readings in the same culture.

The OR of sterile media varied between 310 and 375 mv regardless of the rate of aeration. In inoculated media during the first 24 hr the OR dropped from 344 to 228 mv. With continued incubation and aeration rates of 0.5 and 1 vpm the OR dropped to 80 and 160 mv, respectively, in 96 hours. The streptomycin titers were less than 50 units per ml. With 2 to 6 vpm the OR values were between 200 and 255 mv and the titers were approximately 75 units in the same interval of time.

The ferro-ferricyanide favored more uniform electrode readings without otherwise influencing the reactions.

A23. *The Use of Corn Steeping Liquor in Microbiological Research.* R. WINSTON

LIGGETT, A. E. Staley Mfg. Co., Research Laboratory, Decatur, Ill.

Corn steeping liquor is a valuable nutritive material recently given much publicity by its use in the penicillin fermentation. Earlier it was used as a nitrogen and mineral nutrient for yeast production and was known to the trade as "yeast compound." It has been found valuable as a minor adjunct in many fermentations. Essentially it is an extract of corn solubles under acid conditions, pH 4-4.5, in the presence of dilute sulfurous acid and lactic acid. Laboratory and plant study shows that during processing an active microbial population, chiefly lactic acid bacteria and yeasts, assists in the extraction. It contains approximately 8 per cent nitrogen, amino N/total N 0.5; and is high in essential amino acids and minerals, and most of the B complex vitamins. Its value in antibiotic production is in part at least due to the extensive fermentation it has undergone during the wet corn milling process. In addition to its frequently demonstrated powers of enhancing yields in mold fermentations, it is an effective medium for many bacteria. It may replace peptone as a source of available nitrogen, or beef, yeast malt, or other extracts as essential adjuncts. Specially refined products are available for biological work. Further preliminary processing is desirable for specific uses such as use in a clear agar medium.

A24. *Microbiological Aspects of Penicillin. IX. Cottonseed Meal as a Substitute for Corn Steep Liquor in Penicillin Production.* J. W. FOSTER, H. B. WOODRUFF, D. PERLMAN, L. E. MCDANIEL, B. L. WILKER, AND D. HENDLIN. Merck and Company, Inc., Research Laboratories, Rahway, N. J.

Cottonseed meal is at least as good as corn steep liquor for penicillin production in submerged culture by *P. chrysogenum* strains Demerec X1612 and Wisconsin Q176. Without added chemical precursors, cottonseed meal is considerably superior to corn steep liquor. A number of characteristics of the cottonseed meal medium are discussed. *P. chrysogenum* requires an adaptation to

lactose for most rapid and efficient utilization of lactose and production of penicillin.

A25. *The Effect of Beta Radiation on the Production of Penicillin.* M. W. JENNISON AND J. W. IRVINE, JR., Massachusetts Institute of Technology, Departments of Biology and Chemistry, Cambridge, Mass.

The production of penicillin in the presence of beta radiation was investigated with both surface and submerged (shaker) cultures. The radiation source was radioactive phosphorus, P^{32} , in the form of phosphoric acid added to the production mediums before inoculation, to give activities ranging between 0 and 100 microcuries per ml of medium.

The culture mediums and methods employed were essentially those developed at the Northern Regional Research Laboratory. *Penicillium notatum*, NRRL 1249.B21, and *Penicillium chrysogenum*, NRRL 1951C, were used for surface and submerged penicillin production respectively. Assays were carried out by the standard cup method using *Staphylococcus aureus*, FDA 209P, as the test organism.

The presence of beta radiation in the medium appeared to inhibit penicillin production; at radiation levels below 100 microcuries test samples usually assayed lower than, although within the experimental error (± 25 per cent) of, the control assays. In submerged cultures, amounts of penicillin significantly lower than the controls were not found until an activity of about 100 microcuries per ml of production medium was reached. In surface cultures, activities only up to 4.0 microcuries were tried, and no significant effects were noted.

A26. *Chemical Adjuvants Affecting Penicillin Yields on Synthetic Media.* R. W. STONE, H. T. PATTERSON, AND M. A. FARRELL, The Pennsylvania State College, Department of Bacteriology, State College, Pa.

Several groups of compounds have been tested in shake flasks with *Penicillium chrysogenum*, strain X-1612, to determine the effect on penicillin production. The basal medium was of known chemical composition containing lactose, glucose, acetate, and mineral salts. The amount of antibiotic produced was checked by routine assays with *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella enteritidis*.

The common fatty, hydroxy, and dibasic acids have shown no significant stimulation of penicillin yields. Likewise very little effect has been evident with most of the amino acids used. *L*-Leucine and certain of the sulfur-bearing amino acids have sometimes given increased yields but the effect has not been consistent. A series of aromatic compounds related to phenylacetic acid and phenylethylamine have markedly stimulated penicillin yields. Phenoxyacetic acid and the *meta*- and *para*-halogen derivatives of phenylacetic acid have been quite effective. *P*-nitrophenylacetic and *p*-aminophenylacetic acids increase the activity of the antibiotic for *B. subtilis* and *S. enteritidis*. In most cases the amide derivatives were just as effective or better than the corresponding acids.

A27. *Studies on a Spirochate Found in the Blood of Sick Turkeys.* W. R. HINSHAW AND ETHEL McNEIL, University of California, College of Agriculture, Department of Veterinary Science, Davis, Calif.

A spirochate obtained from the blood of sick turkeys has been proved pathogenic for turkeys and chickens. It stains readily with Tunnick's stain; is soluble in 10 per cent bile, and in 10 per cent saponin. It is loosely spiraled, has an average of 6 spirals, and measures an average of 14 microns in length in stained specimens. Dark-field studies show that it is motile.

The spirochate remains viable in the blood of surviving infected chicks for 3 to 17 days, with an average of approximately 10 days. In adult chickens and turkeys the maximum survival time in the blood of survivors has been 4 days. In infected chicks which have been killed and stored at 0 C, the organisms have remained viable and capable of producing infection for at least 16 days. At the peak of infection the organisms tend to form large clumps in the blood, and become granular immediately before death of the host.

Transmission with infected blood has been possible by the following routes: intravenous, intraperitoneal, intramuscular, intranasal, intraorbital, subcutaneous, and oral. A distinct rise in temperature accompanies infection. The mortality rate has been lower than reported in avian spirochatoxosis in other parts of the world. This is believed to be the first outbreak of spirochatoxosis in turkeys reported in North America.

A28. *Sulfa Drugs in the Control of Shigella gallinarum Infections.* D. FRANK HOLTMAN AND GLADYS FISHER, University of Tennessee, Department of Bacteriology, Knoxville, Tenn.

An investigation was made of the value of sulfa drugs in the control of fowl-typhoid infection. Sodium sulfathiazole, soluble sulfonamide no. II, sodium sulfamerazine, and insoluble sulfamerazine were employed. These agents were administered to young chicks through feed or water in amounts of 0.1 and 0.5 per cent. The drugs checked both naturally occurring outbreaks and experimentally induced infections within two or three days, but the disease frequently reappeared after discontinuance of treatment. In one instance, the disease reappeared five days after the chicks had been removed from a treatment of 14 consecutive days.

Prolonged administration of sulfa drugs in 0.5 per cent concentration gave evidence of retarding the growth and weight of chicks without impairment of appetites. Post-mortem examination of experimentally infected chicks, sacrificed after 10 days on sulfa drug treatment, revealed no gross pathology. That the chicks still harbored *Shigella gallinarum* was proved by the isolation of the organism from the gall bladder. The bacilli were not isolated from other organs of the treated chicks, as was readily accomplished when the disease was permitted to run its course in the absence of sulfa drug treatment.

A29. *Commercial Inoculation of Legume Seed.* FRED S. ORCUTT AND ALMA L. WHITMAN, Virginia Polytechnic Institute, Department of Biology, Blacksburg, Va.

Inoculation of legume seed prior to sale has not been considered to be practical since the available literature indicates that the rhisobia will not remain viable for a sufficient period to be effective. Chemicals used in disinfection of such seeds are difficult to remove and residual amounts may inhibit growth of the desired bacteria. The results of our investigations reveal that a procedure, not involving disinfectants which tend to remain on the seed, may be used, and that inoculation with desirable strains of *Rhizobium* may be satisfactory in that survival times greater than one year may be demonstrated. Therefore, it appears that large-scale commercial inoculation of legume seed may be practical.

A50. Fermentation of Organic Acids by Marine Mud Cultures. R. W. STONE, The Pennsylvania State College, Department of Bacteriology, State College, Pa.

The action of cultures from marine muds on certain organic acids has been studied to determine whether bacteria under simulated marine conditions can produce hydrocarbons or compounds that could be regarded as precursors of hydrocarbons. Mixed cultures were developed by adding marine muds to a sea water medium enriched with a small amount of yeast extract and one of the following compounds: lactic, propionic, palmitic, and glutamic acids, leucine, and phenylalanine. Calculation of carbon balances indicates that for the smaller molecular weight compounds, acetic acid was the primary end product. It was generally accompanied by small amounts of carbon dioxide and higher fatty acids such as butyric. Ether extraction of liquors from the amino acid fermentations has given a constant but very small per cent of neutral material, some of which contains sulfur or sulfhydryl groups. Ether extracts from palmitic acid and phenylalanine dissimilations likewise have contained acetic acid and significant amounts of higher acids. One mud culture was able to convert phenylalanine almost quantitatively to phenylacetic acid.

In all of these studies, the marine cultures have produced acid rather than neutral compounds. The necessary oxygen for this process apparently was obtained by sulfate-reducing bacteria from the sulfate ion present in sea water. The common occurrence of H_2S in active fermentations of this nature is added evidence of the source of oxygen.

A51. Lipid Transformations by Anaerobic Bacteria. WILLIAM D. ROSENFELD AND CLAUDE E. ZOBELL, University of California, Scripps Institution of Oceanography, La Jolla, Calif.

Lipolytic anaerobes have been isolated from muds, water samples, and materials associated with petroleum deposits. Hydrolyzable substrates included glycerides and other esters of fatty acids, as well as more complex oils, and lipolysis was marked at Eh levels often more reducing than -400 millivolts.

The detection of lipoclasts was facilitated by the use of neutral red base, an indicator proposed by Knaysi. The progress of lipolysis was followed by using a Warburg respirometer to measure carbon dioxide evolution from bicarbonate buffer in the presence of fatty acids released by hydrolysis.

Considerable numbers of lipid-utilizing cultures are facultative in their oxygen

requirement. A notable exception is the group of strictly anaerobic sulfate-reducing bacteria. Impure cultures containing sulfate reducers have actively consumed both glycerides and oils as sole carbon sources, although it has been difficult to isolate lipolytic sulfate-reducing colonies. Associative activities may thus be responsible for the transformations observed in these instances. There is evidence to suggest that sulfate reducers utilize end products of lipolysis. The production of hydrocarbonlike substances from lipids has been observed in this laboratory on several occasions. Such syntheses may have resulted from the utilization of fatty acids in an extremely reducing environment.

A32. Effect of Growth of Microorganisms upon Formation of Peroxides, Free Fatty Acids, Aldehydes, and Ketones from Oils with Different Iodine Numbers.
JOSEPH HEJNA AND LESLIE R. HEDRICK, Illinois Institute of Technology,
Department of Biology, Chicago 16, Ill.

The organisms *Pseudomonas fluorescens*, *Serratia marcescens*, *Aspergillus niger*, and *Penicillium italicum* were grown upon a 50 per cent emulsion (with and without the antiozidant NDGA) of the following oils: coconut (iodine number 8 to 10), soya bean (iodine number 130 to 140), and olive (iodine number 170 to 180). All the oils were refined, bleached, and neutral in reaction. At the beginning of the experiments and after an incubation period at 20 C for three intervals of seven days, the substrates were tested for peroxides, free fatty acids (FFA), aldehydes, ketones, and rancid odors.

In general, the growth of the organisms caused an increase in the amount of free fatty acids (FFA); but they produced a decrease in the quantity of peroxides in comparison with the amount formed in the sterile oil control. Ketones were formed only by molds, and only in oils with low molecular weight.

In coconut oil emulsion, most FFA was formed by *Aspergillus*, least FFA by *Pseudomonas*; peroxides, greatest reduction by *Aspergillus* and least reduction with *Pseudomonas*. With soya bean emulsion, most FFA was formed by *Penicillium*, least by *Serratia* (with NDGA); peroxides, greatest reduction by *Pseudomonas* (with NDGA) and least reduction with *Aspergillus*. In olive oil emulsion, most FFA was formed by *Serratia*, least FFA formed by *Pseudomonas*; peroxides, greatest reduction by *Pseudomonas* (with NDGA) and least reduction with *Aspergillus*. There was no correlation between the rancidity detected organoleptically and the results of the chemical tests performed.

A33. The Bacteriostatic Action of Short Chain Fat Acids. ORVILLE WYSS, University of Texas, Department of Bacteriology, Austin, Texas.

Certain of the fat acids exert a bacteriostatic action in a mineral salts glucose medium in addition to the effect observed in the presence of amino acids or peptone. This inhibition is reversed by casein hydrolyzate, by aspartate or glutamate, and by pantothenate. It is exhibited by acetate, propionate, and butyrate but not by formate or valerate or by compounds of chain lengths exceeding 5 carbon atoms. This appears to be another example of the effect of inhibitory analogues on the utilization of metabolites.

- A34. *The Effect of Agar upon the Germicidal Potency of the Quaternary Ammonium Salts.* R. QUISNO, I. W. GIBBY, AND M. J. FOTER, The Wm. S. Merrell Co., Department of Bacteriology, Cincinnati 15, Ohio.

During investigations of death rates of bacteria treated with quaternary ammonium salts, a discrepancy was noted between results obtained with liquid media and results obtained with media containing agar. It appeared probable that agar had partially neutralized the germicide.

To determine the effect of agar upon the germicidal potency of quaternary ammonium salts 0.2 per cent agar was included in the germicide-bacteria mixtures during the test period. Standard germicide tests without agar were included as controls. When the four quaternary ammonium salts were tested in the presence of 0.2 per cent agar, lethal concentrations were found to be three to six times greater than lethal concentrations in the absence of agar. Several investigators have reported a lack of correlation between results obtained with liquid media and results obtained with agar cup plate tests for these germicides. The neutralization effect of agar probably accounts for this discrepancy.

Standard tests for antiseptic potency call for evaluation of creams, ointments, powders, etc., by agar cup plate methods. It is evident that such procedures are inappropriate for preparations which contain quaternary ammonium germicides.

- A35. *The Relation of pH and Quinine to Growth and Disinfection Rates of Escherichia coli.* ISAAC LEWIN AND FRANK H. JOHNSON, Princeton University, Department of Biology, Princeton, N. J.

During the early logarithmic growth phase of *Escherichia coli* in a synthetic medium, a transfer of the cells from neutral to increasingly acid media causes an initial retardation or cessation of growth, or a disinfection, followed by a resumption of growth. This subsequent growth has a much higher apparent activation energy at pH 4.9 than that of controls at pH 6.9.

The rate of disinfection at temperatures above the growth optimum is increased by lowering the pH. The growth-inhibitory and disinfection-promoting effects of 0.0007 M quinine at pH 6.9 are practically eliminated at pH 5.9. At pH 4.9 quinine apparently protects against the initial growth inhibition or disinfection accompanying the transfer of cells from a medium of neutral reaction to one of acid pH. The rate of disinfection at 46.1 C, although accelerated by 0.0007 M quinine at pH 6.9 is retarded by quinine at pH 4.9. The growth-inhibitory and disinfection-promoting actions of the drug are evidently due to the free alkaloid base. The protective effects of quinine at acid pH values occur in other phenomena as well as growth, by a mechanism that is not clear.

- A36. *The Rate of Growth and Disinfection of Escherichia coli in Relation to Temperature, Hydrostatic Pressure, and Quinine.* FRANK H. JOHNSON AND ISAAC LEWIN, Princeton University, Department of Biology, Princeton, N. J.

The growth rate of *Escherichia coli* during the early logarithmic phase in a synthetic medium at neutral pH is limited by a single system in which the activity

increases with rise in temperature, but decreases beyond an optimum at 37–39 C by a reversible denaturation of the protein catalyst. Bacteriostasis without disinfection occurs during brief exposures to 45 C, and growth is resumed at once on cooling to 37 C. Hydrostatic pressures of 1,000 lb/in² retard growth below 35 C but accelerate it above; 5,000 lb cause slight disinfection at low temperatures but greatly retard disinfection at temperatures above 45 C.

The net effect of quinine depends on concentration, temperature, hydrostatic pressure, coenzyme, pH, and oxidizable substrate. Growth inhibition increases with temperature, and is reversible on dilution or cooling. Relatively high concentrations at low temperatures or lower concentrations at higher temperatures cause disinfection. Small amounts of bacterial extracts of co-dehydrogenase I oppose the growth-inhibitory and disinfecting action. Pressure is additive to the effects of quinine at low temperatures but strongly opposes its action at high temperatures. Quinine apparently acts in two ways: (1) promoting a reversible and an irreversible protein denaturation, and (2) specifically blocking hydrogen transfer through the co-dehydrogenase system. The pressure effects indicate large molecular volume increases of reaction or activation, typical of reactions involving proteins.

A37. The Evaluation of Germicidal Agents by an Infection-Prevention Toxicity Method. EARLE H. SPAULDING AND AMEDEO BONDI, JR., Temple University School of Medicine, Department of Bacteriology and Immunology, Philadelphia, Pa.

The infection-prevention (IP) technique of Nungester and Kempf is a valuable procedure for the study of skin disinfectants. The usefulness of this procedure, however, may be increased by including toxicity determinations, which, except for the omission of the culture, are carried out in the same manner as the infection-prevention tests. The highest dilution of disinfectant which prevents pneumococcus infection in one-half of the mice is designated as IP/50. The toxicity end point (T/50) is then determined by finding the lowest dilution which fails to kill one-half of the test mice. IP/50 divided by T/50 yields a number which has been termed the infection-prevention toxicity (IP-T) index. Compounds which are highly bactericidal and relatively nontoxic possess indices of high values.

A chlorinated phenol, two cationic detergents, an organic mercurial, iodine, and tyrothricin have been evaluated by this method. The highest index was obtained with tyrothricin. Representative results will be presented and compared with those obtained with *in vitro* methods and with the toxicity-index procedure of Welch and Hunter. The value and limitations of the infection-prevention toxicity method will be discussed.

A38. Evaluation of Disinfectants by Tests in Living Animals. M. E. PIERCE AND E. B. TILDEN, Northwestern University Dental School, Department of Bacteriology, Chicago, Ill.

The Nungester and Kempf infection-prevention test in mice, with pneumo-

coccus as the test organism, was used to evaluate the potency of a number of common disinfectants. Of these, phenol and iodine, each in 2 per cent aqueous solution, and DC 12 (dimethylbenzylauryl ammonium chloride) in 1 per cent solution, aqueous or tincture, were almost completely effective in killing the pneumococcus. If DC 12 was diluted to 0.1 per cent, however, not all pneumococci were killed, the number of mice lost being 42 per cent with 0.1 per cent aqueous, and 22 per cent with 0.1 per cent tincture. Merthiolate in 0.1 per cent aqueous solution was almost completely ineffective, as had been shown by Nungester and Kempf. With the newer "metaphen disinfecting solution," which contains 4 per cent benzyl alcohol in 0.04 per cent metaphen, the mortality in the mice was 33 per cent, as compared with 86 per cent for the older 0.1 per cent aqueous metaphen. One per cent aqueous metaphen was quite effective, almost as good as 3 per cent saponated cresol. The alcohols, ethyl in 70 per cent concentration and isopropyl (concentrated), were more effective than expected, the mortality in the mice being less than 20 per cent.

Preliminary tests were carried out with some of these disinfectants against *Mycobacterium tuberculosis*, the mixtures used in a standard phenol coefficient test being injected intraperitoneally into guinea pigs instead of being inoculated into a culture medium. Phenol in 1 per cent aqueous solution, saponated cresol in 2 per cent (by volume), and DC 12 in 1 per cent tincture were effective in killing this organism.

A39. *In Vitro* Studies on the Synergic Action of Sulfonamides and Certain Substituted Diphenylalkyl Compounds—A Preliminary Report, ALBERT L. KLECKNER, Pitman-Moore Co., Division of Allied Laboratories, Inc., Department of Pharmaceutical Research, Indianapolis 6, Ind.

A study of the *in vitro* bacteriostatic properties of combinations of sulfanilamide and di-phenthane 70 (2,2'-dihydroxy-5,5'-dichlorodiphenylmethane) suggested the existence of a synergism between sulfonamides and certain closely related substituted diphenylalkyl compounds.

Potential studies were conducted on a strain of *Streptococcus mastitidis* in infusion broth pH 7.6. An equivalent of 12.5 per cent of the minimum bacteriostatic concentration (m.b.c.) of one compound was added to each tube in a serial dilution of the other compound. All tubes, including controls, were incubated for sterility, then seeded with 0.1 ml of a 24-hr culture diluted to yield 1,500 to 2,000 organisms per ml of test substance. Tests were incubated for 96 hours at 37 C. Minimum bacteriostatic concentration recorded was the lowest concentration showing no visible growth.

Addition of 0.08 mg per cent di-phenthane 70 (8 m.b.c.) to sulfanilamide, sulfapyridine, and sulfathiazole reduced the bacteriostatic end points from an average of 256 mg per cent to 1 mg per cent; sulfadiazine from 400 mg per cent to 6.25. Concentrations as low as $\frac{1}{16}$ m.b.c. potentiated sulfanilamide 32-fold. Reversely, sulfonamides potentiated di-phenthane 70 two-to eight-fold. In proteose peptone no. 3 medium no marked potentiation occurred; however, the m.b.c. of sulfanilamide and sulfathiazole averaged 2 mg per cent

compared to 256 mg per cent in infusion broth. Addition of 0.032 mg per cent PABA raised the bacteriostatic end point to 128 mg per cent. Diphenthane 70 did not neutralize the antisulfanilamide action of PABA. Studies are now in progress on the action of certain closely related substituted diphenyl-alkyl compounds, also on the bacteriostatic action on other organisms.

A40. The Effects of Pus on Sulfonamide Activity. L. H. SCHMIDT AND CLARA L. SESLER, Christ Hospital, Institute of Medical Research, Cincinnati, Ohio.

This study dealt with the effects of various lots of pus on the *in vitro* activities of sulfathiazole and sulfanilamide against several bacterial species. The pus was obtained from ten patients and represented a variety of lesions and infecting organisms. Various methods of preparation of the pus were used including filtration through muslin, autolysis, and extraction with heat, acids, alkalis, or ether. The preparations were divided and portions sterilized by boiling, autoclaving, or Seitz filtration. The effects of these preparations upon sulfonamide activity were determined both in artificial media (simple and complex) and in human blood.

With two exceptions none of the lots of pus, irrespective of source or method of preparation, affected the activities of sulfathiazole or sulfanilamide against strains of *Diplococcus pneumoniae*, *Streptococcus hemolyticus*, or *Staphylococcus aureus*. The two exceptions were cases in which the pus had been contaminated with novocaine. These preparations did antagonize the activities of the sulfonamides against the above organisms. In one of these cases pus obtained on subsequent days without the use of novocaine did not have this antagonizing action.

All the preparations, however, appeared to antagonize the activities of these sulfonamides against *Escherichia coli*. This effect may have been due to growth stimulation since the tests with *Escherichia coli* were carried out in a simple medium which afforded much better growth upon the addition of pus.

The above findings provide little support for the conception that pus and tissue debris contain substances which antagonize the common sulfonamides.

A41. The *in Vitro* Potentiating Action of Sulfonamides and para-Aminobenzoic Acid on Penicillin Against Pathogenic Bacteria of Recent Isolation. JORGE VIGOUROUX AND GRACIELA LEYTON, Bacteriological Institute of Chile, Santiago, Chile.

The antibiotic action of penicillin for a number of strains of pathogenic bacteria is reinforced over a wide range by the addition of minute amounts of either sulfathiazole, sulfapyridine, sulfadiazine, or para-aminobenzoic acid (PABA). The incorporation of one of the sulfonamides and PABA in the same solution with the penicillin nullifies the potentiation. Penicillin-sulfonamide mixtures have a more pronounced action on penicillin-sensitive strains, however, when PABA is substituted for the sulfonamide. The sensitivity of the organisms to

penicillin plays no part in the potentiation. The extent to which the antibiotic action of the penicillin is increased is directly related to the intrinsic antibacterial action of the substance under investigation. Occasionally organisms are encountered which are susceptible to mixtures of a sulfonamide and penicillin but resistant to the compounds when applied singly.

In general sulfapyridine has the highest potentiating action. The outstanding exception is with *Brucella*, where sulfadiazine is most active. On the basis of these findings and certain other preliminary studies not to be reported at this time the therapeutic use of combinations of sulfonamides and penicillin in certain infections is indicated.

M1. Factors Influencing the Action of Streptomycin in Vitro. G. L. HOBBY, F. LENERT, AND B. HYMAN, Chas. Pfizer and Company, Biological Department, Brooklyn 6, N. Y.

Streptomycin is an antibacterial agent predominantly effective against gram-negative organisms. Impure preparations are bacteriostatic in action, causing either a decrease in the number of organisms present or at least an inhibition of multiplication of the organisms. True bactericidal action is seldom observed, especially among the enteric organisms.

The sensitivity of a large number of strains of bacteria, belonging to several species, has been tested. The amount of streptomycin necessary to inhibit a given strain may vary from day to day. This difference is due at least in part to slight variations in the number of organisms present, the age and density of the culture, and to the species of organisms involved. Difference in buffer concentration, concentration of enrichment substances present, pH, and other factors influence the sensitivity of an organism to streptomycin to an even greater extent. A statement of the sensitivity of any organism to streptomycin is therefore significant only if expressed in relation to a standard strain.

M2. Studies on the Bacteriostatic and Bactericidal Action of Streptomycin on Bacterium tularensis. S. S. CHAPMAN, LT. (JG), USNR; CORA M. DOWNS; AND S. F. KOWAL, M/SGT., USA; Camp Detrick, B Division, Frederick, Md.

Bacterium tularensis was grown in a peptone medium, described by Snyder and others at Camp Detrick, and the bacteriostatic and bactericidal concentrations of streptomycin were determined. The presence of bacterial growth was determined by turbidity readings, plate counts, and mouse titrations. For a broth inoculum of 5,000,000 organisms per ml, the bacteriostatic concentration of streptomycin was between 0.2 and 0.4 units per ml. One unit per ml sterilized the cultures within 24 hours, while 10,000 units per ml sterilized the cultures in less than 30 minutes. Organisms which grew in the presence of 0.1 and 0.2 units per ml did not appear abnormal, but colonies from tubes containing 0.4 and 0.8 units of streptomycin per ml grew slowly and showed a preponderance of unusually large pleomorphic forms.

M3. Studies on Streptomycin Therapy of Experimental Tularemia in White Mice.

S. S. CHAPMAN, LT. (JG), USNR; LEWIS L. CORIELL, CAPT., MC, AUS;
S. F. KOWAL, M/SGT., USA; W. NELSON, PHM1C, USNR; AND CORA
M. DOWNS; Camp Detrick, B Division, Frederick, Md.

Streptomycin administered to mice in a single dose of 10,000 units per kg at the time of intradermal challenge with 15 to 20 MLD of virulent *Bacterium tularensis* protected 92 per cent of the animals. If therapy was delayed 24, 48, or 72 hours after challenge, the minimal dosage of drug which permitted 80 to 100 per cent survival was 10,000 units per kg given subcutaneously every three hours for ten days. This dosage of drug protected 47 per cent of the animals if therapy was delayed 96 hours after challenge. For well-established infections this dosage must be continued for at least six and preferably for fourteen days to permit good survival.

Dosage of 50,000 units per kg every 3 hr administered for 48 to 72 hr permitted 80 to 100 per cent survival when treatment was initiated 48 hr after challenge. Although the percentage survival was high under streptomycin therapy, some animals were found to harbor latent tularemia infections for as long as 50 days after challenge. After termination of therapy, inoculation of spleens from sacrificed survivors into normal mice showed latent infections to be frequent and persistent in animals which received inadequate drug dosage or adequate drug dosage maintained for an insufficient period of time.

Streptomycin fastness was not demonstrated in cultures recovered from mice harboring the latent infection after inadequate therapy.

Under streptomycin therapy the histological lesions in the skin, liver, spleen, and kidney were less severe and healed readily.

M4. The Ineffectiveness of Streptomycin on Tubercular Infections. GEORGE E. ROCKWELL, Milford, Ohio.

The streptomycin, obtained from Eli Lilly and Company, contained 272 units per mg. Bacteriostatic tests showed that this streptomycin inhibited the growth of a saprophytic tubercle bacillus in dilutions through 1:1,000,000. In bactericidal tests it failed to kill this organism even in a concentration of 1:100,000 for an exposure of 26 hours. Sixteen guinea pigs and four rabbits were inoculated with virulent tubercle bacilli. One-half of the animals were used as controls and the other half were treated for 31 days with streptomycin. They received approximately 14,000 units per kg weight every 24 hours, divided into six doses.

In general the animals receiving the streptomycin lived longer than the controls. At the end of 31 days of treatment some of the treated animals looked healthy while others clinically showed some tubercular involvement. At this time many of the untreated controls were dead and on autopsy showed extensive tubercular lesions. After discontinuance of the treatment with streptomycin, the treated animals began to decline and died at a later date. At the time of their death autopsy showed them to have as extensive tubercular involvement as did the controls.

These experiments indicate that the *in vivo* as well as the *in vitro* action of

streptomycin on the tubercle bacillus is purely bacteriostatic. In a disease such as tuberculosis in which acquired immunity is very meager at its best, it is probable that to be effective a chemical therapeutic agent must be bactericidal in action.

M5. The Sensitivity of Tubercle Bacilli in Vitro to Streptomycin. GUY P. YOUNG AND WILLIAM H. FELDMAN, Northwestern University Medical School, Department of Bacteriology, Chicago, Ill.; Mayo Foundation, Rochester, Minn.

A large number of strains of tubercle bacilli, including human, bovine, and avian types, have been tested *in vitro* to determine the least amount of streptomycin which would completely inhibit subsurface growth. Both stock cultures and recently isolated cultures were included. The majority of the human and bovine strains were found to be inhibited by less than one μg of streptomycin per ml. The majority of the avian strains, on the other hand, were found to be more resistant to the inhibitory action of streptomycin: several cultures required 25 to 50 μg of streptomycin per ml to completely prevent growth. The growth of several cultures of tubercle bacilli isolated from tuberculous patients who had received prolonged treatment with streptomycin was not inhibited by concentrations of streptomycin as high as 1,000 μg per ml. The growth of cultures isolated from the same patients prior to the institution of streptomycin therapy was inhibited by less than one microgram per ml.

M6. Outbreaks of Epidemic Catarrhal Jaundice and Sporadic Cases of Icterohaemorrhagic Spirochetosis in the United States. A. PACKCHIANIAN, University of Texas Medical Branch, Laboratory of Microbiology, Galveston, Texas.

Outbreaks of epidemic catarrhal jaundice (infectious hepatitis) have been studied both from the epidemiological and laboratory standpoints. Special reference is made to epidemics which occurred in Detroit, Michigan; Austin, Minnesota; and Windber, Pennsylvania. Sporadic cases of icterohaemorrhagic jaundice (Weil's disease) in the United States have been reviewed, reference being made to cases which were found during outbreaks of epidemic catarrhal jaundice. Differential diagnosis, including laboratory procedures, of epidemic catarrhal jaundice, icterohaemorrhagic spirochetosis, and yellow fever is discussed and described.

M7. Serological Differentiation of Oral Spirochetes by a Precipitin Test. RUTH H. WICHELHAUSEN AND LUCILLE B. ROBINSON, The Johns Hopkins University School of Medicine, Department of Bacteriology, Baltimore 5, Md.

Serologically specific extracts may be prepared from cultured spirochetes, using dissolution of the organisms by formamide followed by treatment with acid alcohol and acetone. By the use of such extracts in a precipitin test with anti-spirochetal sera it has been possible to place eighteen strains of oral spirochetes

and four strains of alleged *Treponema pallidum* in seven groups. Some of these strains were morphologically and culturally as well as serologically distinct; others were morphologically and culturally indistinguishable but differed in their serological reactions. The extract of one strain of oral spirochetes failed to react even with its homologous antiserum.

M8. *Aerobacter cloacae* as a Possible Factor in the Etiology of Bagassosis. ROY SCHNEITER, WARREN H. REINHART, AND BARBARA H. CAMINITA, Industrial Hygiene Research Laboratory, National Institute of Health, Bethesda, Md., and Industrial Hygiene Section, Louisiana State Department of Health, New Orleans, La.

Bagassosis, a respiratory disease occurring among workers handling sugar cane bagasse, has been attributed to pathogenic fungi and/or their toxic products, to the mechanical irritation of lung tissue by the sharp spicules of bagasse, and to allergy. None of these explanations is completely satisfactory.

The symptoms of bagassosis resemble those of a respiratory illness occurring among workers handling low-grade cotton and caused by the inhalation of an endotoxin liberated by *Aerobacter cloacae* growing in or on the cotton fibers. Bagasse has been reported to contain a toxic material. Furthermore, fresh bagasse offers an ideal medium for the growth of *A. cloacae*. Studies were therefore undertaken to ascertain the presence of *A. cloacae* and its toxin in bagasse.

Thirty-eight samples, representing raw bagasse, old baled or stored bagasse, mill dust, mill air samples, and sawdust from bagasse fiber board, were examined by the serial dilution plate method. *A. cloacae* was demonstrated in five samples of bagasse, one sample of mill dust, and one sample of fiber board sawdust. The plate counts for this type of microorganism ranged from 4,900 to 116,000,000 per gram. The presence of toxigenic strains was confirmed by biochemical methods. The endotoxin liberated by this type of microorganism was demonstrated by Shwartzman tests to be present in sterile saline extracts of five samples of bagasse containing *A. cloacae* and from one sample in which the organism could not be demonstrated.

As a result of these studies it is suggested that *A. cloacae* endotoxin should be considered as one of the possible etiological factors in bagassosis. The results obtained warrant further investigation of this problem.

M9. Fleas Carrying Endemic Typhus *Rickettsiae* Found on Nonmurine Hosts. J. V. IRONS, OLETA BECK, AND J. N. MURPHY, JR., Texas State Health Department, Bureau of Laboratories, Austin, Texas.

Several reports have appeared concerning fleas as natural carriers of endemic typhus. For the most part, these findings have concerned fleas collected from rats. We have accorded epidemiological significance to finding the common cat or dog flea, *Ctenocephalides felis*, harboring typhus rickettsiae on kittens which were intimately associated with five cases of typhus fever. We can now report the recovery of a strain of endemic typhus rickettsiae from a small pool of

Xenopsylla cheopis, the tropical rat flea, taken from a kitten. In addition, one pool each of typhus-infected *Ctenocephalides felis* was taken from two young opossums and three puppies. Although sera from some of the hosts of these fleas were reactive in low titers by the complement-fixation test with murine typhus antigen, we have not yet succeeded in demonstrating the presence of typhus rickettsiae in tissues of any naturally infected animal other than the rat and mouse.

M10. Polyvalent Antigens for the Serodiagnosis of Salmonella Infections. OSCAR FELSENFELD, Mt. Sinai Research Foundation, Chicago 8, Ill.

Polyvalent "O" and "H" antigens were prepared according to the method of Welch and Stuart for spot agglutination tests. The polyvalent "O" antigen was composed of the antigens of *S. schoëttmuelleri*, *S. hartford*, *S. enteritidis*, *S. london*, and *S. senftenberg*. The polyvalent "H" antigen contained the antigens of *S. paratyphi*, *S. schoëttmuelleri* var. *java*, *S. typhimurium*, *S. hartford*, *S. muenchen*, *S. enteritidis*, *S. london*, and *S. selandia*. This combination of antigens covers a part or the total of the antigenic structure of *Salmonella* strains usually encountered in America. Comparison with the results of tube agglutination tests using sera with known agglutinin content have shown that positive spot agglutinations with serum dilutions of 1:40 and higher have to be considered significant. The advantages of this spot test lie in its simplicity and the rapidity with which it can be performed.

M11. The Use of Polyvalent Serum for the Rapid Presumptive Identification of Salmonella Cultures. WM. D. BARCUS, R. B. MITCHELL, AND R. H. BROH-KAHN, AAF School of Aviation Medicine, Department of Bacteriology, Randolph Field, Texas.

During an extensive survey for the detection of *Salmonella* carriers, it became expedient to possess a means for the rapid presumptive identification of *Salmonella* cultures. Felsenfeld had described the use of a polyvalent serum for this purpose. Through the offices of the O.S.R.D., Dr. P. R. Edwards of the Kentucky Agricultural Experiment Station prepared and standardized, for use by the Army Air Forces, three polyvalent sera which contained agglutinins against all of the known, naturally occurring antigens recognized in *Salmonella* cultures. These sera were used either in dilutions of 1:5 for rapid slide O-agglutination tests with emulsions prepared directly from colonies of non-lactose-fermenters or in dilutions of 1:500 for tube agglutinations for detection of the characteristic H antigens. Preliminary observations indicated that all known *Salmonella* organisms were agglutinated by one or more of the three sera when used in the slide agglutination technique. All motile *Salmonella* cultures were agglutinated in the tube test. The sera appeared to be quite specific insofar as paracolons and other non-lactose-fermenters failed to react with the technique used.

The successful use of these sera requires no particular skill and they are well

adapted to use by the routine clinical laboratory. They afford a simple method by which clinicians can be informed, at the earliest moment, of the presence of a *Salmonella* organism in a stool culture.

M12. The Occurrence of Paracolon Organisms with Salmonella XXXVIII Antigen.

VIOLA MAE YOUNG, Mt. Sinai Research Foundation, Chicago 8, Ill.

Paracolon organisms were tested for the presence of *Salmonella* XXXVIII antigen, which was described by Edwards and Hughes as part of the antigenic structure of *Salmonella inerness*. This antigen was found in three paracolon strains, the first isolated from meningitis case, the second from the stool of a veteran who returned from Africa with chronic diarrhea, and another from the feces of a patient during a food-poisoning outbreak of unknown origin. The biochemical reactions of the three observed strains agreed in that they did not ferment lactose or salicin, did not form indole, were M.R.-positive, and utilized citrate as the sole source of carbon. It is noted that while paracolon strains containing other *Salmonella* antigens were often isolated from healthy persons, paracolon organisms with XXXVIII antigen were not found in the stools of individuals displaying no symptom of disease.

M13. Mucoid Polysaccharide Production, Encapsulation, and Colony Morphology of Carrier Strains of Group A Streptococci. ROBERT M. PIKE, Southwestern Medical College, Department of Bacteriology and Immunology, Dallas, Texas.

The observation of a group A streptococcus carrier rate of 25 per cent in the throats of well children indicated the desirability of examining these strains for the presence of certain characteristics which may be associated with virulence. Sixty-four per cent of 229 carrier strains produced amounts of mucoid polysaccharide which were detectable by the turbidity method of Seastone; 43 per cent showed capsules in Wright's stained smears of young cultures, while 7 per cent produced mucoid colonies on blood agar and feathery colonies in 0.2 per cent agar. A similar incidence of these characteristics was observed among 54 strains of group A streptococci isolated from infections.

The quantitative estimation of mucoid polysaccharide by reading photoelectrically the turbidity which develops in broth culture supernatants on the addition of 10 per cent horse serum at pH 4.2 was found to be a more sensitive and less variable test for the presence of capsular substance than the microscopic observation of capsules, and much more reliable than the morphology of surface colonies or of deep colonies in soft agar.

By the fermentation of lactose, mannite, and salicin, 270 of the 283 strains were classified as *Streptococcus pyogenes*, eleven as *S. infrequens*, and two as *S. scarlatinae*.

M14. The Fate of Leprosy Bacilli in Tissues Cultivated from Leprous Lesions.

JOHN H. HANKS, Leonard Wood Memorial Laboratory, Cullion, Philippines.

Biopsied nodules and skin from neural lesions provided naturally infected cells from susceptible persons. Fragments from these tissues were explanted into plasma films in test tubes and the cells were nurtured by repeated applications of serum and embryo juice. The cultures were incubated at 34 C. When cell colonies required division, they were transplanted into new tubes and thus maintained for periods of three to seven months.

The fibroblasts in the early outgrowth from nodules were stuffed with bacilli. By injecting dilute India ink into nodules prior to biopsy, outgrowths were obtained in which carbon and bacilli occurred in similar proportions of the cells. As cell populations expanded during cultivation, the proportion of cells with these particles declined at similar rates. After two or three months the curves for bacilli began to fall more rapidly than those for carbon, indicating disappearance of the bacilli. Destruction of the bacilli was hastened by stimulating more rapid growth of the cells or in atmospheres containing twice the usual pressure of carbon dioxide. Media which reduced the growth rate to about one-half of that in serum and embryo juice decreased the destruction of bacilli.

Fibroblasts from neural lesions, when allowed to ingest large numbers of microorganisms, rapidly reduced the bacilli to acid-fast debris. Fibroblasts from lepromatous cases are indifferent to the same or greater concentrations of bacilli.

The relation of these observations to several clinical features of leprosy will be discussed.

M15. Effect of Penicillin Aerosol on Pneumococcus Pneumonia in Rats. FAITH P. HADLEY, PHILIP HADLEY, ALICE P. McILROY, AND ANGELA M. LAURENT, Western Pennsylvania Hospital, Institute of Pathology, Pittsburgh, Pa.

The aim of this study was to ascertain the therapeutic and prophylactic effects of penicillin, administered by inhalation of a penicillin-containing mist, on experimental pneumococcus pneumonia in rats. Typical lobar pneumonia, showing all the pathological stages of human pneumonia, was produced by intra-bronchial inoculation of approximately 60,000 mucin-suspended, type I pneumococci. The penicillin mist, produced by nebulization of a penicillin solution containing 40,000 units per ml, was blown into a treatment chamber in which 10 rats were exposed simultaneously for 15-minute periods. Of 106 rats for which treatments, by various modifications of dosage, were begun 18 hr after inoculation (a time at which consolidation of the inoculated lobe had already taken place), 80, or 75 per cent, survived. Of 137 control, untreated rats, 30, or 22 per cent, survived. In some experiments, in which the technique of inoculation and the details of therapeutic procedure were developed to a more favorable point, the survival rate of treated rats was 90 to 100 per cent, and 0 to 10 per cent for the untreated.

When treatment was begun one hour after inoculation, and repeated at two-hour intervals up to seven hours, 73 per cent of 30 treated animals survived, compared with a 13 per cent survival of 30 untreated controls. When treatment

was repeated at intervals from one to 28 hours after inoculation, 100 per cent of 20 treated and none of 20 control animals survived.

Inhalation treatments given one hour or less, previous to inoculation, had no effect in preventing the development of pneumonia produced by mucin-suspended pneumococci. When the organisms were suspended in broth, without mucin, and inoculated intrabronchially, inhalation of penicillin aerosol previous to inoculation prevented infection.

M16. Specific Complement-fixing Diagnostic Antigens for Neurotropic Virus Diseases. CARL J. DEBOER AND HERALD R. COX, Lederle Laboratories, Inc., Virus Research Division, Pearl River, N. Y.

Complement-fixing antigens for certain neurotropic virus diseases have been accepted generally as useful diagnostic agents. Various investigators have described the preparation and use of such diagnostic antigens. In most instances such materials were found to give specific fixation in the presence of homologous antisera. However, all the preparations reported by previous investigators suffer from the serious defect that they give false positive reactions in the presence of syphilitic serums when the Kolmer technique is employed.

Details are described of a simple method for preparing specific complement-fixing diagnostic antigens for such neurotropic virus infections as Eastern and Western equine encephalomyelitis, St. Louis encephalitis, and Japanese B encephalitis. The method is essentially that recently reported by Wolfe, Van der Scheer, Clancy, and Cox for the preparation of specific rickettsial diagnostic antigens and consists of using certain fat solvents for the extraction of virus-infected tissues completely dried from the frozen state.

These preparations possess the significant property of not giving rise to false positive reactions even in the presence of highly positive Wassermann serums (Kolmer technique). The method apparently may be readily applied to any type of infected tissue shown to be rich in virus.

M17. The Virus Neutralization Test in Primary Atypical Pneumonia and Other Acute Respiratory Diseases. MONROE D. EATON AND WILLIAM VAN HERICK, California State Department of Public Health, Virus Laboratory, Berkeley, Calif.

Neutralization tests with paired acute phase and convalescent serums from 213 persons with various respiratory diseases were done according to the published method with the new virus from atypical pneumonia isolated and propagated in chick embryos. A significant increase (fourfold or greater) in virus-neutralizing antibodies was found in 62 per cent of 84 cases of primary atypical pneumonia. Increases in neutralizing antibodies were found in 20 of the 27 cases with cold agglutinins and in 22 of 24 patients who developed agglutinins for the indifferent streptococcus (strain no. 344).

Serums from 77 patients with undifferentiated acute upper respiratory infections were tested. Of these, 19.5 per cent showed a significant rise in titer of virus-neutralizing antibodies. As controls 52 pairs of serum specimens from a

group of respiratory diseases which included 12 bacterial pneumonias, 5 pneumonias of the psittacosis group, and 35 cases of type A influenza were also examined. Only one of these showed a significant increase in neutralizing antibodies against the virus isolated from atypical pneumonia. Other results to be presented indicate that the virus under consideration has caused sporadic cases of pneumonia or local outbreaks of acute respiratory disease in every year since 1941 and in several regions of the United States.

M18. The Neutralization of the Mouse-adapted Poliomyelitis Virus by the Sera of Children. CHARLES F. PAIT, JR., JOHN F. KESSEL, AND PHILIP GROSSMAN, University of Southern California, Department of Bacteriology, Los Angeles County General Hospital, Los Angeles, Calif.

The purpose of this study is to assay sera from children of different age groups for their capacity to neutralize the poliomyelitis virus. The experimental method consisted of a neutralization test using eight mice per serum or serum dilution tested. Equal quantities of virus and serum were mixed and incubated *in vitro*, then tested for virus by intracerebral inoculation in eight 3-week-old mice. Virus pools were prepared from infected mouse spinal cords and were titrated by the 50 per cent end point method. A dose was used in the test which produced 75-85 per cent mortality. Any serum protecting five or more of the eight mice was considered as a neutralizing serum. If three or fewer mice survived, the test was read as negative. Ninety-one sera were so tested. All positive sera were titrated in four dilutions ranging from 1:8 to 1:1,000. Tests were controlled by similar groups of animals inoculated with the virus, both neutralized and unneutralized by known positive and negative sera, and mixed with the buffer used for making the virus and serum dilutions. The serums of the newborn reflect the antibody level of the mother. The percentage of positive sera in each age group then decreases so that between the ages of one to four years only about 25-30 per cent are strongly positive. After this there is a steady increase in the strongly positive sera up to the age of ten years and over, at which time the ratio of positive to negative sera is essentially that of the adult.

M19. Allergic Encephalomyelitis in Monkeys in Response to Injection of Normal Monkey Cord. ISABEL M. MORGAN, Johns Hopkins University, Poliomyelitis Research Center, Baltimore 5, Md.

By the use of Freund's adjuvant technique in monkeys an allergic reaction has been induced to normal monkey spinal cord injected subcutaneously with falba, paraffin oil, and heat-killed tubercle bacilli. This results in a characteristic clinical reaction of ataxia, spasticity, and disorientation often associated with blindness appearing from two to seven weeks following injection. Seven of twelve monkeys thus inoculated have shown such a reaction, as well as eight of nine monkeys injected with adjuvants plus poliomyelitis-infected cord. None of eight control monkeys injected with adjuvants plus saline has shown any abnormal sign or pathological change in the central nervous system; nor any of another series of eight injected with adjuvants plus other organs. The patho-

logical reaction consists of intense foci of perivascular and extravascular infiltration irregularly disseminated throughout the brain and spinal cord, showing a mixture of lymphocytes and polymorphonuclear leucocytes including a varying proportion of eosinophils. There is often focal necrosis and hemorrhage.

M20. Studies on Equine Encephalomyelitis in Michigan. GORDON C. BROWN, University of Michigan, School of Public Health, Virus Laboratory, Ann Arbor, Mich.

During 1943 an epidemic of equine encephalomyelitis occurred in Michigan which involved the greatest number of cases ever to be reported from that state. In view of the reported isolation of a strain of Eastern virus from the brain of a horse by Dr. S. D. Kramer of the Michigan State Department of Health, a study was undertaken to determine the identity and possible reservoir of the virus.

Sera were obtained from healthy horses and from others convalescing from the disease and from 71 wild fowl including geese, pheasant, peafowl, turkeys, ducks, swans, and cranes inhabiting a bird sanctuary in the center of the epidemic area. Neutralization and complement-fixation tests were performed with these sera and the antigens of Eastern and Western equine encephalomyelitis virus. The interpretation of the results with the horse sera was somewhat complicated by the fact that some of the horses had been vaccinated. Nevertheless, it was clear that the sera contained antibodies for both Eastern and Western viruses and that their presence was not due to previous vaccination.

The Eastern strain of virus was neutralized by the sera of three fowl, a Formosan pheasant and two wild turkeys. The Western strain was neutralized by three different sera, from two blue geese and a Canada goose. Complement fixation, when positive, corroborated these results.

It is indicated clearly that both the Eastern and Western strains of equine encephalomyelitis were active in Michigan and that certain wild fowl may serve as a reservoir for these viruses.

M21. Further Observations on the Virus of Encephalomyocarditis. JOEL WARREN AND JOSEPH E. SMADEL, Army Medical School, Division of Virus and Rickettsial Diseases, Washington, D. C.

Helwig and Schmidt, of the U. S. Army, recently recovered a transmissible filterable agent from a chimpanzee which induced a disease in mice characterized by paralysis and myocarditis. This agent was sent to the Army Medical School for further study.

The agent becomes highly neurotropic when maintained by serial intracerebral passage in mice. Encephalitis develops rapidly and mice die within 24 hours. Titration end points of 10^{-3} to 10^{-4} are obtained when infected brain is injected intraperitoneally or intracerebrally into young or old mice. No macroscopic evidence of myocarditis occurs in mice dying rapidly, but an extensive acute focal necrotizing myocarditis, visible grossly, occurs when death is delayed. Histological changes in the brain are characterized by widespread necrosis of

nerve cells when death occurs early and extensive cellular infiltrations after a protracted disease. Necrosis of Purkinje cells of the cerebellum is striking.

Hamsters developed encephalitis and myocarditis after inoculation of virus. Guinea pigs, rabbits, and a rhesus monkey showed only febrile reactions following intracerebral injection. Each of these species developed specific neutralizing antibodies during convalescence. Few mice survive infection but these subsequently resist reinfection.

Attempts to demonstrate immunological relationships between the virus of encephalomyocarditis and other filterable agents have been unsuccessful. The following viruses or antisera were employed: St. Louis, Japanese, West Nile, Semliki Forest, Russian spring-summer, and Louping Ill encephalitis; Eastern, Western, and Venezuelan encephalomyelitis; herpes; lymphocytic choriomeningitis; Theiler's (GD VII); poliomyelitis (Lansing); and influenza "A" and "B." The small size of the Florida agent eliminated the elementary body viruses from consideration.

M22. Isolation of a New Virus from Two Fatal Pneumonia Cases. JOSEPH ZICHIS AND HOWARD J. SHAUGHNESSY, Division of Laboratories, Department of Public Health, Chicago, Ill.

During the winter of 1944 two viruses were isolated from two fatal cases of pneumonia in Chicago. One was isolated from each case and they were regarded as different viruses until studies indicated that they were identical. This infectious agent has been termed the Illinois virus.

The virus was isolated by inoculating mice by the intranasal route with a suspension of the lung tissue. The virus kills mice when it is administered by the intracerebral, intraperitoneal, intranasal, and subcutaneous routes. Following each method of inoculation, L.C.L. bodies are produced. The virus passes through Berkefeld N or W filters.

Immunological comparisons were made between the Illinois virus and the psittacosis, ornithosis, S.F., and meningopneumonitis viruses. These tests showed that the Illinois virus was not immunologically identical with any of the above viruses.

M23. Isolation of Psittacosislike Viruses from Chicago Pigeons. JOSEPH ZICHIS, HOWARD J. SHAUGHNESSY, AND CATHERINE LEMKE, Division of Laboratories, Department of Public Health, Chicago, Ill.

During the summer of 1945, the serum of each of 200 pigeons caught in Chicago was tested by the complement-fixation test with antigens prepared from the Illinois and psittacosis viruses. Of the 200 sera tested, 73 gave positive reactions with both antigens. One was positive only with the Illinois virus antigen and one only with the psittacosis virus antigen.

The tissues of 27 pigeons were tested for the presence of psittacosislike viruses. Portions of the spleen, liver, and lungs of each bird were emulsified in broth and inoculated into Swiss mice by the intracerebral and intranasal methods. Psitta-

cosislike viruses were isolated from 6, or 22.2 per cent, of the pigeons. Preliminary studies on the identification of these viruses indicate that they apparently fall in the psittacosis group but not all of them are psittacosis or ornithosis viruses.

M24. Studies on Antigenic Relationships Within the Psittacosis-Lymphogranuloma Group of Viruses. ELIZABETH ST. JOHN AND F. B. GORDON, University of Chicago, Department of Bacteriology and Parasitology, Chicago, Ill.

The experiments reported here constitute a continuation of the study under way in this laboratory of antigenic relationships within the psittacosis-lymphogranuloma group of viruses by means of antisera prepared in chickens. Antisera were produced by repeated intraperitoneal injection with the viruses of mouse pneumonitis (Chicago), feline pneumonitis, meningopneumonitis, lymphogranuloma venereum, and Ann Arbor virus. Neutralization tests were performed using intracerebral or intranasal inoculation of mice. The strains against which the sera were tested included, in addition to the above, the viruses of human pneumonitis (SF), psittacosis, and the Illinois virus.

Evidence of antigenic cross relationship among the strains was obtained only in the case of mouse pneumonitis and Ann Arbor viruses. Antimouse pneumonitis serum neutralized Ann Arbor virus to full titer but anti-Ann Arbor serum neutralized mouse pneumonitis virus to approximately half titer, suggesting that these two strains are closely related but not identical. In no other instance did an antiserum neutralize a strain other than the one against which it was prepared, although not all possible combinations have been tested.

Each of the strains against which an antiserum was made, with the exception noted above, appears to be antigenically distinct from the others used in these experiments when examined by this technique.

M25. Effect of in Vitro Cultivation on the Pathogenicity of West Nile Virus. HILARY KOPROWSKI AND EDWIN H. LENNETTE, Yellow Fever Research Service, Rio de Janeiro, Brazil.

The West Nile virus was propagated in a medium consisting of serum-tyrode's solution and minced chick embryo without the CNS. Pathogenicity of the virus at various passage levels was determined by titration of cultures in three-day-old mice subcutaneously, in 8-, 14-, 21-, and 28-day-old mice intraperitoneally, in 28-day-old mice intracerebrally, and on two occasions in Syrian hamsters by the intracerebral and intraperitoneal routes.

The lethal effect of the virus for mice and hamsters by the intracerebral route remained essentially unchanged during 79 passages—the mortality ratios and LD₅₀ end points showed no significant differences between low and high passage virus. The average survival time of mice inoculated with high passage virus, however, was somewhat longer than that of mice which received low passage virus.

In contrast, the ability of the virus to invade the CNS after peripheral inoculation was markedly altered, the extent of the change in mice depending upon the passage level of the virus and the age of the animals; the younger (and more

susceptible) the mice, the number of passages which was required to produce attenuation. After 68 passages, the virus still retained its full capacity to kill three-day-old mice, while its ability to kill eight-day-old mice was reduced and its ability to kill mice 14 or more days of age was essentially abolished.

Prolonged cultivation rendered the virus avirulent for hamsters by the intra-peritoneal route.

M26. Intraocular Infection with the Viruses of Ornithosis and Feline Enteritis.

CHARLES A. EVANS, University of Minnesota, School of Medicine, Department of Bacteriology and Immunology, Minneapolis 14, Minn.

Ornithosis virus injected into the anterior chamber of the eyes of three rabbits caused clouding of the cornea and purulent conjunctival exudate. In sections, acute inflammation was found in the tissues of the filtration angle and in adjacent corneal and uveal tissues, and purulent exudate in the anterior chamber. Characteristic inclusions (L.C.L. bodies) were common in macrophages and fibroblasts of the inflamed tissues and exudate. A very few inclusions were present in corneal endothelium. The corneal opacity (edema) probably resulted largely from toxicity of the virus inasmuch as infection of corneal endothelium was minimal at the time the corneal changes developed.

Intraocular injection of feline enteritis virus into nine cats in several experiments resulted in no apparent abnormality of the eyes. All cats showed symptoms characteristic of systemic infection (panleucopenia, anorexia, profound asthenia). In sections of the inoculated eyes, inclusion bodies were found sparsely distributed in many kinds of cells (bipolar and ganglion cells and pigment epithelium of the visual retina, pigmented and nonpigmented epithelium of the ciliary body, and fibroblasts in the iris and ciliary body). Apparently this virus infected cells at the site of inoculation but failed to spread significantly in the eye.

From these data and previously published experiments, the ocular reactions to representative viruses are—fox encephalitis in foxes, dogs, raccoons: infection of corneal endothelium causing opacity; influenza in rabbit: no infection, corneal opacity due to toxicity; ornithosis in rabbit: corneal opacity due to infection plus toxicity; feline enteritis in cat: limited infection of many kinds of cells, no readily visible ocular change.

M27. The Chemotherapy of Experimental Psittacosis Infection (Strain 6BC).

ROBERT L. EARLY, 1ST LT., CWS, AUS, AND HERBERT R. MORGAN, CAPT., MC, AUS, Rockefeller Institute for Medical Research, New York 21, N. Y.

Sodium sulfadiazine or penicillin were found to be effective agents in the treatment of experimental psittacosis infections in mice infected with psittacosis virus (strain 6BC) by the intravenous or intraperitoneal routes. However when the intracerebral or respiratory routes of infection were used, the results obtained with sulfadiazine were superior to those obtained with penicillin. Streptomycin had no significant effect.

M28. Effect of Minerals on Susceptibility of Mice to Theiler's Encephalomyelitis. ABRAHAM C. LICHTSTEIN, CONRAD A. ELVEHJEM, AND PAUL F. CLARK, University of Wisconsin, Departments of Medical Bacteriology and Biochemistry, Madison, Wis.

The present report is concerned with the influence of dietary minerals on the susceptibility of Swiss mice to Theiler's encephalomyelitis. The basal synthetic diets and vitamin additions were identical with those of our previous studies, the only alteration being in the mineral components. Swiss mice bred in our laboratory were used in all experiments and split litter technique employed. The GDVII strain of virus was given by intracerebral route using an inoculum of 0.03 ml. All animals were observed twice daily after inoculation for signs of flaccid paralysis; the experiments were terminated 28 days after administration of the virus. The minerals studied were K, Na, P, Ca, Mg, and Cl, and in all approximately 1,100 mice were employed.

Essentially no influence on susceptibility to Theiler's encephalomyelitis was found by varying the level of Ca, Mg, or Cl in the diet; slight effect was noted with Na; and striking results were obtained with K and P. The following is an indication of the results obtained by varying the level of K in the diet: K deficient (32 per cent paralyzed), 5 per cent of optimum K requirement (51 per cent paralyzed), 15 per cent of optimum K (79 per cent paralyzed), optimum K requirement (91 per cent paralyzed). Equally striking results were obtained with P: 5 per cent of optimum P requirement (33 per cent paralyzed), 15 per cent of optimum P (48 per cent paralyzed), 30 per cent of optimum P (58 per cent paralyzed), optimum P (80 per cent paralyzed). It was impossible to distinguish between paralysis due to P deficiency or to virus invasion when the P was completely absent from the ration; hence, 5 per cent of optimum P requirement was used as the basal diet.

M29. Therapeutic Use of Specific Typhoid Bacteriophage. WALTER E. WARD, University of Southern California, Department of Medical Bacteriology, and Los Angeles General Hospital, Communicable Disease Unit, Los Angeles, Calif.

In the absence of specific methods for treatment of typhoid fever a reinvestigation was begun several years ago of the possibilities of bacteriophage therapy.

Laboratory work was first initiated by producing infections in mice. Treatments were given intravenously with specific and nonspecific V bacteriophage. Extension of the work to human infections was made. In the latter, diagnoses were made by blood culture and the organisms typed by the method of Craigie and Yen. High titer specific bacteriophages were prepared in a chemically defined medium capable of supporting growth of *Eberthella typhi* in the V form.

The results show that infected mice treated specifically with bacteriophage have a mortality of about 5 per cent while infected mice treated with bacteriophage inactive for the infecting strain have a mortality approaching 100 per cent. Infected mice treated with specific bacteriophage demonstrate an increase in the content of bacteriophage in the blood.

Human cases of typhoid fever treated with specific bacteriophage show a crisis within 12-24 hours with negative blood cultures, normal temperatures, and definite evidence of clinical improvement. There is as yet no definite evidence of bacteriophage multiplication in treated human cases.

All evidence supports the belief that specific typhoid bacteriophage is therapeutically effective in experimental animal infections because of bacteriophage activity. The mechanism of action of specific bacteriophage in treated human cases of typhoid is not clear. The end results are, however, spectacularly good.

M30. The Effect of Booster Vaccination on Protective Humoral Antibodies Against Dysentery in Children. MERLIN L. COOPER, JACK TEPPER, AND HELEN M. KELLER, The Children's Hospital Research Foundation and Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

This study was made under contract with the Office of Scientific Research and Development. It was part of a larger study to determine the possibility of using vaccines of *Shigella* for active immunization of human subjects against dysentery.

Earlier studies with monovalent *Shigella* vaccines had demonstrated stimulation of the production of protective humoral antibodies against dysentery in children and that in some instances these persisted to a significant degree for 52 weeks. Earlier studies with polyvalent *Shigella* vaccines also demonstrated that such stimulated the production of protective humoral antibodies against each of the component antigens and that these persisted to a significant degree for 30 to 52 weeks.

The present report deals with the effect of booster vaccination on recalling protective humoral antibodies against dysentery in children. A group of five children were given three subcutaneous injections of a monovalent *Shigella* Boyd 88 vaccine at intervals of one week. The passive mouse-protective power of their sera was determined over a period of 52 weeks, by which time it had gradually returned to zero. Each child was then given three subcutaneous injections of a vaccine similar to, but ten per cent as concentrated as, that used for the primary vaccination. Sera obtained after each booster dose showed rapidly increasing content of protective humoral antibodies indicating that booster doses of antigen were effective in recalling these antibodies.

M31. Preparation and Evaluation of an Irradiated Toxoid from the Toxin of Shigella dysenteriae. SARA E. BRANHAM AND KARL HABEL, National Institute of Health, U. S. Public Health Service, Bethesda, Md.

Search for an immunizing agent in bacillary dysentery has involved studies with vaccines, with various fractions of dysentery bacilli, and in the case of the toxin-producing Shiga strains, with toxoids. This paper describes the preparation of a toxoid by the ultraviolet radiation of a liquid Shiga toxin. This toxin was prepared by a modification of the method described by Farrell and Ferguson. It was irradiated by the use of the lamp described by Oppenheimer and Levinson.

Although the toxin was affected by exposures as short as 0.1 second, or less, complete inactivation required exposures of from 11 to 15 seconds. Complete detoxification was determined by intravenous injection of mice and of rabbits. A dose harmless for mice may often be lethal for rabbits.

Although the toxicity of these preparations was destroyed, the antigenicity remained high. Mice vaccinated with this toxoid were completely protected against a challenge dose of toxin fatal for all control mice. Moreover these vaccinated mice withstood injection with 100 to 10,000 fatal doses of living Shiga cultures (in mucin) intraperitoneally.

Control groups of mice were vaccinated with living irradiated, heat-killed, formalin-killed, and phenol-killed suspensions of Shiga bacilli, and with extracts of the microorganisms. With none of these preparations was the immunity obtained as marked as with the irradiated toxoid.

Rabbits were immunized with these irradiated preparations. Their sera protected mice to a marked degree against both toxin and living culture.

M32. Chemical Detoxification of Dysentery Antigen. F. W. BARNES, M. M. DEWEY, S. S. HENRY, AND M. H. LUPFER, Children's Hospital Research Foundation, and Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

Alteration of the antigens of *Shigella paradysenteriae* so as to reduce toxicity without equivalent lowering of protective power has been a major objective in the problem of preparing dysentery vaccines for protection of troops and civilians in the war. Previous attempts to accomplish this have been unsuccessful.

Since July, 1945, however, it has been apparent that the antigen of type Z (III), with which we have worked, can be affected in such a way as to produce a net reduction or 90 per cent or more in toxicity on the basis of a standard protective value.

Progress in the investigation has been measured by a mouse test which gives the toxicity-protection ratio of such an altered vaccine. In this test, toxicity is measured by 24-hr weight loss in groups of mice receiving geometrically decreasing doses of the vaccine. Protection is measured by a constant, challenging dose of homologous virulent organisms given to the same groups of mice one week later. The reduction in toxicity is accomplished by treating bacterial suspensions or purified antigens with 2 per cent hydrogen peroxide in the presence of limited amounts of heat or of ultraviolet light over a period of two or more hours. The addition of sodium caprylate in 0.05 M concentration and of sodium chloride in 0.25 M concentration prevents injury to the protective power of the antigen during the procedure.

M33. Immunization of Humans and Animals with Gas Gangrene Toxoids. ALFRED A. TYTELL, MILAN A. LOGAN, ALICE G. TYTELL, AND JACK TEPPER, University of Cincinnati, Medical College, Department of Biological Chemistry, Cincinnati, Ohio.

Alum-precipitated toxoids of *Clostridium perfringens* and *Clostridium oedematiens* have been prepared. These toxoids have been demonstrated to be effective in producing a measurable and protective titer of antibodies in mice, guinea pigs, pigeons, dogs, goats, and humans. In animals good protection against large doses of toxin and culture have been obtained. Protection of animals against heterologous as well as homologous strains of culture was demonstrated. Titers have been achieved in humans which in smaller animals have shown good protection against both toxin and culture challenge. In humans one dose of toxoid of *C. perfringens* was inadequate. With two doses 40-70 per cent of the subjects showed a positive antitoxin response. When a third dose was given three to nine months after the second dose, excellent titers were obtained in 95-100 per cent of the individuals tested. Response to two doses of toxoid of *C. oedematiens* was good, but response to a third dose was excellent.

Divalent toxoids (*C. perfringens* and *C. oedematiens*) have been prepared by mixing adequate amounts of the alum toxoids. These divalent toxoids have shown very adequate protective response in animals. Trials in humans have shown good response to both antigens. There appeared to be no significant suppression of either antigen.

MS4. Production of Potent Inactivated Vaccines with Ultraviolet Irradiation. V. Active and Passive Immunization with Lymphocytic Choriomeningitis Vaccine. ALBERT MILZER AND SIDNEY O. LEVINSON, Michael Reese Research Foundation, Serum Center, Chicago, Ill.

Previous attempts to immunize mice against the lymphocytic choriomeningitis virus have been successful only when active virus was employed. We also were unable to immunize mice or monkeys with infected guinea pig brain or spleen suspensions inactivated by heat or formalin. By means of a new technique of ultraviolet irradiation described elsewhere, 4 per cent uncentrifuged brain tissue suspensions infected with J.P. strain of lymphocytic choriomeningitis virus were completely inactivated by irradiation for 0.3 seconds. Mice immunized with three intraperitoneal 0.5-ml injections of irradiated vaccine given at weekly intervals resisted at least 200 LD₅₀ of virus given twenty days after the first dose of vaccine. Rhesus monkeys immunized in a similar manner resisted approximately 10 LD₅₀ of virus. Hyperimmune monkey serum prepared by repeated injections with irradiated suspension followed by active lymphocytic choriomeningitis virus was employed in the treatment of normal mice injected intracerebrally with 5 LD₅₀ of virus. There was definite protection when serum was given as long as 48 hr after infection with 50 per cent of the animals surviving, while the mortality rate in the controls was 100 per cent.

MS5. Psittacosis Vaccines Prepared from Chick Embryo Tissues. J. C. WAGNER, 1st Lt., SNC, AUS; G. MEIKLEJOHN, Lt., MC, USNR; L. C. KINGSLAND, Lt., MC, USNR; AND H. W. HICKISH, 1st Lt., CWS, AUS; Camp Detrick, Frederick, Md.

The purpose of this study was to develop a chick embryo tissue vaccine effective for immunization of animals against the 6BC strain of psittacosis. Various types and modifications of the current chick embryo tissue vaccine production techniques were investigated. A new procedure is reported in which the lyophilization of yolk sac material during the extraction yielded vaccines of unusually high immunizing potency. Mice injected subcutaneously or intraperitoneally with the test vaccine were subsequently challenged by the respiratory or intracerebral routes to determine the degree of protection afforded. The results indicate that some protection against respiratory and intracerebral challenge can be obtained with killed virus preparations.

M36. The Immunization of White Rats Against Tularemia. LEWIS L. CORIELL, CAPT., MC, AUS; CORA M. DOWNS; HENRY T. EIGELSBACH, ENS., H(S), USNR; GIFFORD B. PINCHOT, LT., USNR; BARBARA OWEN, LT. (JG), USNR; BETTE HAMILTON; G. R. SPENCER, ENS., USNR; LUTHER BUCHELE; Camp Detrick, Frederick, Md.

The experimental immunization of white rats against tularemia is described, using vaccines prepared from *Bacterium tularense* grown in embryonated chicken eggs, peptone broth, gelatine hydrolyzate broth, and glucose cysteine blood agar. Vaccines prepared from virulent and from avirulent strains of *B. tularense* conferred about equal protection to subsequent challenge with a virulent strain. Subcutaneous vaccination protected rats against challenge by subcutaneous, intraperitoneal, or intranasal routes. Rats were not passively protected against fatal infection by serum from immune rats, and no bactericidal activity of immune serum was demonstrated *in vitro*. Studies on the pathogenesis of tularemia in normal and immune rats are presented.

M37. Studies on the Nature of Antibodies Produced in Vitro from Bacteria with Hydrogen Peroxide and Heat. EDWARD C. ROSENOW AND FRANK H. JOHNSON, California Institute of Technology, The Gates and Crellin Laboratories of Chemistry, Pasadena, Calif.

Further studies on the production of "thermal antibodies" *in vitro* have shown that, by heating 10,000,000,000 organisms per ml, without the addition of sensitizing protein, for one hour at 17 pounds' steam pressure, in NaCl solution, pH 2.5 to 4.5, containing an oxidizing agent such as H_2O_2 (1.5 per cent initially), agglutinin, precipitin, and protective titers are obtained that are as great or greater than by autoclaving corresponding suspensions, without H_2O_2 , for 96 hours. These thermal antibodies are highly stable at acid pH, distillable on boiling, dialyze slowly through cellophane against NaCl solution, and readily pass through diatomaceous, Seitz, or fritted glass filters. They are more species-than type-specific, compared to natural antibodies.

When thermal antibodies are added to protein solutions, e.g., serum or globulin at room temperature, agglutinin titers diminish sharply. When similar mixtures are heated 10 min at 65 C at atmospheric or 10,000 lb p.s.i. hydrostatic pressure, then cooled slowly, agglutinin titers greatly increase, properties resembling

natural antibodies are acquired, i.e., they are no longer or much less distillable, and they become nondialyzable. Furthermore, type specificity is increased. The possibility is suggested that diffusable antibody may be formed *in vivo* as well as *in vitro* by oxidation of bacterial antigens, which then combine with normal globulin to produce nondiffusable antibody globulin.

M38. An Immunological Study Involving Carcinogenic Hydrocarbons. EVELYN L. OGINSKY, O. N. ALLEN, AND HUGH J. CREECH, Department of Bacteriology, University of Maryland, College Park, Md.; Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia 30, Pa.

The purposes of this study were (1) to determine whether carcinogenic hydrocarbons act as haptene groups when conjugated with proteins, and (2) to ascertain whether the immune sera are specific for the homologous hydrocarbon.

Horse serum albumin coupled by carbamido linkage with 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene, and human serum albumin coupled with 1,2-benzanthracene were used as antigens. Rabbits were immunized by multiple intravenous and intraperitoneal injections totaling approximately 450 mg of the conjugated or control native protein. Precipitin reactions with the resulting antisera were read macroscopically, and were confirmed quantitatively by micro-Kjeldahl analyses of the washed precipitates.

The results indicate that the conjugated proteins had gained new specificity due to the determinant group. 1,2-Benzanthracene was found to be more effective as a haptene than the other two hydrocarbons. Antisera showing the best results were those formed by injection of 1,2-benzanthryl-10-carbamido horse serum albumin. Although these sera reacted most strongly with the antigen used for injection, they demonstrated haptene group activity by reacting, to a lesser degree, with 1,2-benzanthracene conjugates from human and bovine serum albumin, and with 1,2,5,6-dibenzanthracene and 3,4-benzpyrene conjugates from bovine serum albumin. Quantitative analyses confirmed the observation that such cross reactions were greatest at a definite ratio of antigen and antiserum which varied for the individual sera. Immunological activity of the albumin portion of the conjugates decreased with introduction of the hydrocarbon groups.

The haptene activity of these compounds makes desirable further research on protective immunization of animals against carcinogenesis due to various hydrocarbons.

M39. The Mechanism of the Adhesion of Dog and Human Platelets to Bacteria. RALPH B. HOULIHAN, University of Virginia, School of Medicine, Department of Preventive Medicine and Bacteriology, Charlottesville, Va.

This report deals with an investigation of the formation of "infective" thrombi in septic processes in man and animal. Dog platelet-rich plasma, and serum and defibrinated blood to which isolated platelets had been added, were rotated with various pathogenic and nonpathogenic staphylococci and streptococci.

Microscopic examination of these systems revealed intense adhesion of platelet clumps to all bacteria tested. Platelets which had been washed free of plasma constituents and suspended in saline were stable and did not adhere to bacteria.

In contrast to the marked platelet-bacteria adhesion observed in dog blood, human platelets exhibited little or no adhesiveness when tested under identical conditions. The addition to human platelet-rich plasma of saline extracts of human heart, heart valve, and vein tissues induced adhesion of platelets and bacteria in rotating systems. Purified gamma globulin, albumin, fibrinogen, and extracts of human placenta and artery tissues, however, were not effective in producing mixed clumping of platelets and bacteria.

M40. Studies on Biologically Universal Serum Reactions (Nonsyphilitic) with Lipid Antigens. REUBEN L. KAHN, Clinical Laboratories, University Hospital, University of Michigan, Ann Arbor, Mich.

It was previously reported from this laboratory that serodiagnostic tests for syphilis can be so modified as to give very nearly 100 per cent precipitation reactions with sera from nonsyphilitic human beings and animals. These reactions, evidently of a biologically universal nature, were obtained by the use of especially prepared, excessively sensitive tissue extract antigens. Present studies indicate that biologically universal reactions can be obtained also with serodiagnostic antigens provided optimal salt concentrations are employed with appropriate temperatures and incubation periods in the performance of the tests. The lipid extracts employed in this study included standard and sensitized Kahn antigens and cardiolipin antigen developed by Pangborn. False positive reactions obtained with serodiagnostic tests for syphilis show optimal reactivity under conditions similar to those employed in eliciting biologically universal reactions. Hence, it is believed that the latter reactions may be the basis for false positive reactions given by serodiagnostic tests.

M41. A Serum Precipitation Reaction in Rheumatic Fever and in Other Diseases. A. G. WEDUM, University of Colorado, School of Medicine, Department of Bacteriology, Denver 7, Colo.

A precipitation resembling a strongly positive Kahn test appeared when the serum of a patient in the prerheumatic (poststreptococcal) state or phase II was mixed with the serum of a patient in the acute state of rheumatic fever or phase III. Convalescent serum also contained this phase II substance, which has been designated "A substance" to differentiate it from the "B substance" present in phase III. These substances were present less frequently in serums from patients with nasopharyngitis, atypical pneumonia, and other diseases. Whether the reaction is caused by serum lability or a system of closely related autoantigens and antibodies is unknown. There is a possibility that the occasional unfavorable clinical response to blood transfusion in patients with rheumatic fever may be correlated with the presence of these substances in donor and recipient.

M42. *The Reaction of Paramecia in Specific Antiserum.* JAMES A. HARRISON AND ELIZABETH H. FOWLER, Temple University, Department of Biology, Philadelphia 22, Pa.

The reaction of paramecia in specific antiserum differs in certain respects from the reaction commonly observed when bacterial cultures are examined in antisera. The agglutinative reaction, though very infrequently observed with the paramecia, usually takes the form of rosette development. The paramecia show distinctive surface changes in antiserum which are not readily observable, if they occur, in the bacteria. These surface changes include an early development of a gelatinous and sticky antigen-antibody product in the form of small balls at or near the end of the cilia followed by a collection of a similar gelatinous product between the cilia. A prominent feature of the early reaction is a sticking together of the cilia at the ends where the balls are formed. Frequently it occurs that a part of the precipitate which collects in the ciliary zone is extruded and swept to the rear to form a sea-anchor arrangement which trails behind the animal as long as it retains some motility. When extensive amounts of precipitate are formed there often occurs a shrinkage and crenation of the cell. The interior components are not frequently afflicted by antibody; when they are, the most prominent change is an acute dilation and paralysis of the contractile vacuoles.

M43. *The Mechanism and Nature of the Complement Reaction as Related to Toxin-Antitoxin Dissociation.* SARAH E. STEWART AND A. EARL VIVINO, Georgetown University Medical School, Department of Bacteriology and Pharmacology, Washington 7, D. C.

Toxin-antitoxin dissociation has been demonstrated by various procedures, e.g., destruction of toxin by acid hydrolysis with release of antitoxin, or digestion of antitoxin using proteolytic enzymes with release of toxin. Using the lecithinase reaction, shown by the toxin of *Clostridium perfringens*, we have been able to demonstrate toxin-antitoxin dissociation by release of acid-soluble phosphorus from weak alcoholic lecithin substrate with a neutral toxin-antitoxin mixture. Complement has been found to inhibit this dissociation tenfold, heated or inactivated complement being as effective as the unheated complement. Calcium is essential for lecithinase activity, so was used in all the tests performed, and it may be that the calcium was also responsible for the activation of the complement. This activation of complement confirms Lieberman's observation on the production of artificial complement by adding to heat-inactivated rabbit serum a methyl alcoholic solution of calcium oleate.

M44. *The Influence of Temperature on Phagocytosis.* DORALEA R. HARMON, CHRISTINE ZARAFONETIS, AND PAUL F. CLARK, University of Wisconsin, Department of Medical Bacteriology, Madison, Wis.

Exudative polymorphonuclear leukocytes of guinea pigs, rabbits, and mice were tested *in vitro* in phagocytic systems containing normal homologous serum and *Staphylococcus aureus* at five-degree temperature intervals over the range

of 22–42 C. An increase in phagocytosis at each successive temperature was observed as measured quantitatively by the Hamburger technique of counting the percentage of phagocytes ingesting bacteria. A rough phagocytic index was likewise determined as a check. Tubes containing the various phagocytic systems were slowly rotated end over end for ten minutes in a water bath set at the desired temperatures. Slides were made from each tube and two slides (200 leukocytes per slide) at each temperature were examined. Labeling of the slides was covered with tape to rule out personal bias.

Experiments to determine the effect of previous sensitization of staphylococci with normal serum at different temperatures were devised. Previous opsonization at 37 and 40 C followed by phagocytosis at both temperatures in each instance showed that both opsonization and phagocytosis were favored by the higher temperature, the latter to a greater degree than the former. Similar tests at 22 and 37 C indicated that phagocytosis was enhanced by the higher temperature while opsonization was impaired by it.

M45. Methods for the Irradiation of Biological Liquids with Wavelength 2537 Å.

ALEXANDER HOLLAENDER, J. W. OLIPHANT, AND H. L. ANDREWS, Industrial Hygiene Research Laboratory and Division Infectious Diseases, National Institute of Health, Bethesda 14, Md.

In laboratory practice the biological liquids most commonly sterilized by ultraviolet irradiation are blood serum, plasma, suspensions of various parts of the developing chick embryo, brain suspensions, and other highly proteinaceous materials whose absorption spectra show a typical maximum around 2800 Å and continuous absorption at wavelengths shorter than 2500 Å. Bacteria, fungi, and most viruses usually inactivated show a maximum of sensitivity at 2650 Å, close to the nucleic acid absorption band. The availability of inexpensive and efficient 2537 Å sources has made the inactivation of infectious disease agents in biological liquids by ultraviolet a promising method.

Since radiation of 2537 Å has relatively low penetrating power for biological liquids, conditions for adequate irradiation would require (1) that the material be free of dense aggregates (tissue pieces, clumps, and precipitates) and (2) the assurance that all particles receive adequate and equivalent irradiation. This paper will illustrate simple equipment for the irradiation of small quantities and apparatus for irradiating material in a continuous flow, give some of the results obtained, and discuss the limitations of the method.

M46. The Initial Distribution of Air-borne Bacteria in the Host. ADA MAY AMES AND W. J. NUNGESTER, University of Michigan, Department of Bacteriology, Ann Arbor, Mich.

The findings reported were obtained in a study of the defense mechanisms of the animal body against air-borne organisms. The experimental procedures have been divided into two main groups: first, those aimed at determination of the normal distribution pattern of inhaled organisms in the respiratory tract,

esophagus, and stomach; second, those attempting to vary that pattern. The studies were carried out by exposing various species of animals in a closed chamber to an aerosol of spores of a nonpathogenic tracer organism, *Bacillus globigii*. The animals were then sacrificed and the organisms present in the nasopharynx, esophagus, trachea, lungs, and stomach determined.

The results indicate that the number of spores recoverable from the various parts of the respiratory and digestive tracts of normal animals is related to species, cloud concentration, and to the time elapsing between exposure and sacrifice. For a given species, the distribution is not appreciably affected by sex, weight, or the interval of time after feeding. The differences noted between species can be correlated with differences in tidal air and with size of the respiratory passages.

The distribution pattern of inhaled organisms can be varied to produce an increasing number reaching the lungs by light anesthesia, nasal vasoconstriction, and early acute pneumonitis. On the other hand, extensive pulmonary consolidation, deep anesthesia, and the moribund state reduce the relative number found in the lungs.

M47. Cultural and Serological Studies on Granuloma Inguinale. WOLCOTT B. DUNHAM AND GEOFFREY RAKE, Squibb Institute for Medical Research, Division of Microbiology, New Brunswick, N. J.

Studies have been made with a strain of *Donovania granulomatis* (Katherine Anderson), the presumed causative agent of granuloma inguinale. The 92nd egg passage was inoculated on yolk beef heart infusion agar slants. The strain was carried for eight passages on this medium and then transferred to Levinthal beef heart infusion agar slants. An antigen was prepared from the fifth passage on this medium. Complement-fixation tests on sera from cases of granuloma inguinale and from other infections indicate a specific immunological relationship between granuloma inguinale and the organism cultivated on an artificial medium.

M48. A Simple Medium for Maintenance of Meningococci. MAX LEVINE, LT. COL., SN. C., AUS, AND A. R. THOMAS, JR., COL., M. C., USA, Brooke General Hospital, Clinical Laboratory Service, Fort Sam Houston, Texas.

A medium consisting of beef extract broth, to which were added 1 per cent corn starch, 0.03 per cent dextrose, 0.02 per cent KCl, 0.01 per cent CaCl₂, and 1.5-2 per cent agar, was found to support a vigorous growth of all cultures of meningococci encountered in this laboratory. Whereas all strains died in 3-4 days at icebox, room, or incubator temperatures when grown on blood agar, they survived on the above starch salt glucose medium for 31 days at 37 C and at room temperature (25-28 C) and for 6 days in the icebox. The cultures of meningococci on this medium were good antigens for agglutination. Gonococci grew well on this medium after primary isolation.

M49. Use of the Chick Embryo for the Enhancement of Virulence of Hemophilus pertussis. L. J. LEWIS, National Drug Co., Research Department, Philadelphia, Pa.

The work of Goodpasture, in growing various organisms in the chick embryo, suggested the possibility of enhancing the virulence of *Hemophilus pertussis*. It is known that the organism gradually loses its virulence for mice when grown on artificial media. The phase, however, is not always changed. Various suspensions of the organism (48-hr cultures on Bordet-Gengou agar) were inoculated directly into 11-day-old chick embryos and incubated for 24 hours. The lungs of the embryos were harvested and cultured on Bordet-Gengou agar. The resulting growth was harvested, checked for purity, suspended in 4 per cent mucin and inoculated into mice. This procedure was repeated in further serial egg passages with the following results: Strains in a 1.5 B/ml suspension which were avirulent to mice killed 1/5 after 1 egg passage, 3/5 after 2 egg passages, 4/5 after 5 egg passages, and 5/5 after 10 egg passages.

M50. The Cultivation of Rickettsia orientalis in Fertile Hens' Eggs. CARL F. CLANCY AND HERALD R. COX, Lederle Laboratories, Inc., Virus and Rickettsial Research Division, Pearl River, N. Y.

Early efforts to produce a vaccine against *Rickettsia orientalis* infection indicated that a richer growth of rickettsiae in the yolk sac membrane was required. Compared with the rickettsiae of epidemic typhus, Rocky Mountain spotted fever, and American Q fever, the infectivity of scrub typhus rickettsiae is relatively low for seven-day-old fertile hens' eggs. By comparison of microscopic smears, the death rate of infected eggs, and titration of infected yolk sac membranes in mice as a measure of the degree of infection, experiments were carried out to increase the infectivity of the virus for eggs. The effect of temperatures above and below the optimal 95 F, generally used for epidemic typhus rickettsiae, on infected eggs was observed. A comparison of various fluids as diluents for egg inocula showed minor advantages in the use of liver extract, 10 per cent normal rabbit serum, and skim milk, but no pronounced change in the death rate of infected eggs was effected.

Since Pinkerton and Bessey showed that riboflavin-deficient rats were more susceptible to endemic typhus, experiments were made to determine if a similar situation existed in respect to *R. orientalis*. Fertile eggs deficient in riboflavin were infected with scrub typhus rickettsiae and were found to be as resistant to infection as the normal control eggs. Similarly, mice on riboflavin-deficient diet were found to be no more susceptible than normal animals.

M51. The Effect of Enzyme Inhibitors, Nitriles, and Substitution Compounds on the Growth of Vaccinia Virus. RANDALL L. THOMPSON, Western Reserve University, Department of Bacteriology, Cleveland 6, Ohio.

The Maitland tissue culture technique affords a means for the determination of the effect of chemical agents on the growth of viruses. From a study of a series of compounds, information was obtained concerning the growth require-

ments of the vaccinia virus as well as an indication as to which substances may have therapeutic value. Cyanide, azide, atabrine, proflavine, hydroquinone, and dinitrophenol inhibit multiplication of vaccinia virus in a concentration of 1×10^{-5} ; menadione and benzimidazole are effective at 1×10^{-4} . Desthio-biotin is without effect. Several compounds were tested in mice to determine their action on the course of vaccinia infection.

RT1. Basic Training in Bacteriology. WILLIAM BURROWS, University of Chicago, Chicago, Ill.

RT2. Bacteriological Training for Professional Students. W. J. NUNGESTER, University of Michigan, Ann Arbor, Mich.

RT3. Graduate Training in Bacteriology. J. HOWARD MUELLER, Harvard Medical School, Boston, Mass.

RT4. The Teaching of Viral and Rickettsial Diseases. F. B. GORDON, University of Chicago, Chicago, Ill.

RT5. The Teaching of Mycology as a Part of Medical Bacteriology. DONALD S. MARTIN, Duke University, Durham, N. C.

RT6. Enlivening and Clarifying the Instruction in Bacteriology by the Use of Audio-visual Aids. HARRY E. MORTON, University of Pennsylvania, Department of Bacteriology, Philadelphia 4, Pa.

The many improvements in the science of teaching were put to test and brought to the attention of the public by the demands for rapid instruction of large numbers of individuals during the recent war. Prospective students know about some of these methods; they will rightly expect the best methods of instruction to be used in the schools and colleges in which they enroll. Teachers of bacteriology must use the most efficient methods of teaching in order to produce what is expected of them in the face of an ever increasing amount of subject matter and in an already crowded curriculum. At the present time there are more aspects to and more interest in bacteriology than at any time in the history of the science. Publishers of textbooks are planning on the correlation of audio-visual aids with material in the textbooks. Producers of various types of teaching films are demanding pictures of microorganisms for their films. The Society of American Bacteriologists needs and has a central clearing place for such visual aids. Various types of visual aids will be demonstrated and their use discussed.

RT7. Bacteriology at the University of Michigan. MALCOLM H. SOULE, Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

RT8. Bacteriology at Michigan State College. WARD GILTNER, School of Veterinary Medicine, Michigan State College, East Lansing, Mich.

RT9. Bacteriology at Wayne University. HARRY L. CLARK, Department of Bacteriology, Wayne University School of Medicine, Detroit, Mich.

RT10. Bacteriology in the City of Detroit, Department of Health. FRED M. MEADER, Kalamazoo State Hospital, Kalamazoo, Mich.

RT11. Bacteriology in the Hospitals and Clinical Laboratories in the Detroit Area. WILLIAM L. BRÖSIUS, Department of Health, Detroit 1, Mich.

- RT12. *Water Bacteriology in the Detroit Area.* WILLIAM M. WALLACE, Filtration Plant, Water Works Park, Detroit 15, Mich.
- RT13. *Bacteriology in the Dairy Industry in the Detroit Area.* ARCHIBALD R. WARD, 1986 Waverly Avenue, Detroit 6, Mich.
- RT14. *Bacteriology in the Michigan Department of Health.* GEORGE D. CUMMINGS, Michigan Department of Health, Lansing 4, Mich.
- RT15. *Geographical and Seasonal Distribution of Influenza.* MONROE D. EATON, Virus Laboratory, California State Department of Health, Berkeley, Calif.
- RT16. *Occurrence of Influenza A and Influenza B at Fort Bragg.* ALTO E. FELLER, Commission on Acute Respiratory Diseases, Regional Hospital, Section 2, Fort Bragg, N. C.
- RT17. *Protective Effect of Vaccination against Influenza B.* THOMAS FRANCIS, JR., University of Michigan, Ann Arbor, Mich.

A study was made of the incidence of influenza B in vaccinated and unvaccinated units at the University of Michigan during an epidemic in the winter of 1945. The results can be discussed in relation to the characteristics of the virus encountered.

- RT18. *Reactivation of Overneutralized Influenza Virus.* A. P. MCKEE AND WILLIAM M. HALE, State University of Iowa, Department of Bacteriology, Iowa City, Iowa.

Numerous attempts have been made to reactivate neutral mixtures of virus and antibody, influenza virus and its antibody not excepted. Electrophoretic dissociation, enzyme digestion, simple dilution, and centrifugation have been employed for this purpose. In experiments previously reported, where the virus-antibody ratio was clearly designated, reactivation of the virus occurred only when the mixture was "apparently" neutral. In the experiments summarized below the mixture of influenza virus, type A (PR8), and its antibody was at least sufficiently neutral so that the virus could not be reactivated by dilution, enzyme digestion, or serial mouse lung passage.

The method employed to accomplish reactivation was the addition of concentrated, heat-inactivated homologous virus to a mixture of virus and antibody which had been in contact previously for one hour at room temperature. The concentrated inactive virus was permitted to react with the mixture for two hours at room temperature before the mice were inoculated. Each batch of concentrated, inactivated virus was proved to be inactive by three serial mouse lung passages.

The following information was ascertained as a result of our experiments: (1) That a neutral mixture of influenza virus, type A (PR8), could be reactivated. (2) That a mixture of the same virus and antibody, overneutralized at least one hundred times could be reactivated. (3) That 10 per cent of the virus present in a neutral mixture could be reactivated by this method. (4) That concentrated, heat-inactivated influenza virus type B (Lee) would not reactivate a neutral mixture of influenza virus type A (PR8) and its antibody.

RT19. *Studies on the Toxicity of Influenza Viruses.* WERNER HENLE AND GERTRUDE HENLE, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

It has been reported previously that influenza viruses when injected by "non-infectious" routes may cause severe lesions in various organs of mice, although propagation of the agents could be demonstrated only in the respiratory tract. Thus, intravenous or intraperitoneal injection of virus led to severe damage of liver, spleen, and other organs; intracerebral injection, to convulsions through destruction of the ependymal lining of the ventricles.

This toxic property has been studied extensively, particularly with regard to (a) optimal conditions for obtaining toxic preparations and quantitative aspects of the reaction; (b) differences in the toxic activity of various strains of influenza virus; (c) the nature of the toxic agent as determined by high speed centrifugation, adsorption onto and elution from red blood cells; (d) the stability of the toxic property, as tested in mice in comparison with the infectivity of the preparation for chick embryos, upon storage at 4 C, or upon treatment with various physical and chemical agents; and finally (e) the role of toxicity in problems of immunity to influenza as studied by neutralization tests with immune sera and by active immunization tests in mice. Possible application of this property of influenza viruses to the assay of vaccines in mice will be discussed.

RT20. *Influenza in Europe During the Winter of 1945-46.* JONAS E. SALK, University of Michigan, School of Public Health, Ann Arbor, Mich.

A watch for early indications of an influenza epidemic was established in the American Occupation Zone in Germany beginning in early December, 1945. Through the middle of February, 1946, no evidence of any epidemic of influenza was noted in either civilian or military populations in this area. However, throughout the period in question sporadic cases of influenza A as well as influenza B were detected. During the same interval epidemics of influenza B were reported in Belgium, France, and England. Certain interesting features of these occurrences will be worthy of comment.

The nature and degree of respiratory disease was observed in the port areas disembarking troops from the United States where an outbreak of influenza B was occurring. Also, the relative incidence of respiratory disease in Army and Navy personnel in the London area was ascertained.

RT21. *Biochemical, Biophysical, and Serological Properties of Purified Influenza Virus.* W. M. STANLEY, Rockefeller Institute for Medical Research, Princeton, N. J.

Fundamental studies have been made of the biochemical, biophysical, and serological properties of purified influenza virus preparations obtained from infectious extra-embryonic fluids of chick embryos and from perfused lungs of infected mice. Following removal of an impurity possessing a very high intrinsic viscosity, an isoelectric point more acid than that of the virus and a sedimentation constant less than that of the virus, it was found that the purified virus was

homogeneous in the ultracentrifuge and in the electrophoresis apparatus. The virus activity was found to be associated solely with particles about 100 m μ in diameter, and composed of protein, nucleic acids, lipid, carbohydrate, and about 60 per cent by weight of water. These particles regardless of their source possess a common antigenic structure which is characteristic of influenza virus, but also possess an antigenic structure which is characteristic of the host from which the virus originated. This finding is of importance in connection with the nature of the structure of viruses.

Conditions for obtaining optimum amounts of virus in allantoic fluids have been determined and the accuracy of different methods of estimating virus concentration has been established. Different methods for the purification, concentration, and inactivation of influenza virus have been evaluated to aid in the large-scale production of influenza vaccines. A Sharples centrifuge method was superior to other methods for purification and concentration of influenza virus. The product obtained consisted mainly of virus, whereas other methods yielded products consisting of only about 20 per cent or less of virus. Yield of virus and maximum concentration of virus obtainable by the centrifuge method were considerably greater than those obtainable by other methods. A method for the large-scale production of a centrifuge-type influenza vaccine has been developed and is now in use.

RT22. Present Specifications for Commercial Streptomycin. WILLIAM A. RANDALL, HENRY WELCH, CLIFFORD W. PRICE, AND VELMA L. CHANDLER, Food and Drug Administration, Washington 25, D. C.

On November 20, following the request of a joint board of the Army and Navy, the Food and Drug Administration in co-operation with the several manufacturers interested in the preparation of commercial streptomycin, prepared the present tentative minimum specifications for control of the standards of identity, strength, quality, and purity of streptomycin. The Army-Navy specifications with the recent allocation order of the Civilian Production Board require that tests and assays be performed on all lots of streptomycin manufactured, for potency, sterility, pyrogens, toxicity, moisture, pH, histaminelike substances, and its effect on growth of several specific bacteria.

Because of certain inherent undesirable characteristics of streptomycin, additional studies and development of new methods are imperative. The drug has already been shown to produce skin reactions, kidney damage, and impairment of the eighth cranial nerve, and control of these factors through proper pretesting is essential. The histaminelike action of the drug appears not to be inherent in streptomycin since it may be reduced or eliminated in the processing of this antibiotic. Its exclusion, however, is essential.

Further studies of the assay organism, the media, and the optimum pH for testing are necessary before final methods can be established. Procedures for determining pyrogens, moisture, and pH are at present similar to methods formulated for certification of penicillin and these appear to be satisfactory.

However, the repeated use of rabbits for pyrogen testing of streptomycin will need study because of the possibility of liver and kidney damage caused by repeated injection of crude lots of this drug.

Development of more precise methods for detecting contamination of streptomycin with the toxic products of other species of *Actinomyces* is highly desirable. The present "bacterial spectrum" method does not give results that are reproducible, in spite of the fact that a streptomycin standard is used for comparative purposes. A discussion of the development of modifications of the present methods will be given.

RT23. The Inactivation of Streptomycin and Its Practical Applications. SELMAN A. WAKSMAN, WALTON B. GEIGER, AND SAMUEL R. GREEN, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology, New Brunswick, N. J.

The antibacterial activity of streptomycin can be largely or completely neutralized or antagonized by various chemical agents. These include glucose and certain other sugars, an anaerobic environment, certain sulfhydryl compounds, and ketone reagents. In some cases, as in the action of sugars or the anaerobic environment, the effect on streptomycin can be traced to the acidity produced under these particular conditions. However, in the effect of cysteine, cevitamic acid, and of ketone reagents the inhibition of streptomycin activity may be associated with the blocking of the active grouping in the molecule of the streptomycin. The inactivation of the antibacterial properties of streptomycin by the blocking of a single group in its molecule cannot be attempted at present due to its rather complicated structure. Until the chemistry of streptomycin is more clearly elucidated, it is difficult to present a suitable theory that would account for the various effects of streptomycin inactivation.

RT24. The Pharmacology of Streptomycin. HANS MOLITOR, Merck Institute for Therapeutic Research, Rahway, N. J.

In contrast to penicillin, streptomycin possesses definite toxic properties, although its margin of safety considerably exceeds that of all chemotherapeutic agents other than antibiotics. Some of the toxic manifestations following its parenteral, particularly intravenous, administration, such as nausea, vomiting, headache, flushing of the face, and fainting spells, may definitely be ascribed to a histaminelike impurity; pure streptomycin is free from these effects. Other side reactions such as prolonged elevation of body temperature, arthralgia, and skin rashes may be due to impurities or abnormal sensitivity of the patient. The most serious complication consists of neurotoxic signs, such as vertigo, locomotor disturbances, and particularly temporary or permanent impairment of the eighth nerve. These toxic phenomena can also be produced in certain animal species. It is not known whether they are due to the active principle of the drug or to impurity. The parenteral toxicity in animals varies greatly with different batches; there is apparently no close correlation between acute

lethal toxicity in animals and incidence of unfavorable side reactions in man. Large doses of streptomycin produce in animals a fatty metamorphosis in the liver and less frequently in the kidney; these changes seem to be reversible.

Streptomycin is readily absorbed upon parenteral administration, but only poorly when given by mouth. It is rapidly eliminated from the blood stream and excreted through the kidneys, making necessary frequent administration for the maintenance of therapeutically effective concentrations in the blood. Drug fastness may rapidly be developed *in vitro* as well as *in vivo*. This necessitates the maintenance of high blood and tissue levels and cautions against oral administration in systemic therapy.

RT25. Present Knowledge of the Chemotherapeutic Properties of Streptomycin.

DONALD R. NICHOLS AND WALLACE E. HERRELL, Mayo Clinic, Division of Medicine, Rochester, Minn.

This paper deals with a discussion of the selective antibacterial activity of streptomycin. It is based on a clinical study of approximately 100 cases in which streptomycin has been used in the treatment of a variety of infections. Dosage, methods of administration, and consideration of toxicity are discussed. The significance of the development of resistance on the part of the infecting organism *in vivo* is carefully considered.

RT26. The Relationships of the Gram-negative, Nonsporeforming, Peritrichous Bacteria to Nonmotile Bacteria. ROBERT S. BREED, New York State Experiment Station, Geneva, N. Y.

At the period when the Society of American Bacteriologists became actively interested in formulating an outline classification of bacteria, Winslow *et al.* (1917) placed the bacteria listed above in a single family *Bacteriaceae*, with a separate family *Lactobacillaceae* for similar gram-positive rods. In the final report by Winslow *et al.* (1920), these two families were united into a single family *Bacteriaceae*. Buchanan (1918), in his classification, included pseudomonads with many gram-positive and gram-negative, nonsporeforming rods in the subtribe *Bacterinae*. Bergey, who never regarded the difference between polar and peritrichous flagellation as particularly significant, kept most of the gram-negative and gram-positive, peritrichous and polar flagellate, nonsporeforming bacteria together in family *Bacteriaceae* in the four editions of his *Manual* published between 1923 and 1934.

Meanwhile, Rahn (1937) pointed out the naturalness of narrower groupings and suggested family *Enterobacteriaceae* for coliform-dysentery-typhoid and related bacteria, and *Parvobacteriaceae* for the more highly specialized parasites and pathogens in the genera *Pasteurella*, *Hemophilus*, *Brucella*, and *Dialister*. These families were accepted in the 5th edition of Bergey's *Manual* (1939), two additional genera (*Malleomyces* and *Noguchia*) being added to the family *Parvobacteriaceae*. *Actinobacillus*, *Bacteroides*, and *Fusobacterium* are being added to the family for the 6th edition. *Achromobacter*, *Flavobacterium*, and *Alcaligenes* have also been organized into the family *Achromobacteriaceae*. This includes

salt and fresh water, soil, and related bacteria that are inactive on sugars. It is believed that recognition of these more homogeneous families will aid in developing a clearer conception of the relationships of these bacteria.

RT27. The Origin of the Name Parvobacteriaceae. OTTO RAHN, Cornell University, Laboratory of Bacteriology, Ithaca, N. Y.

A large majority of the species in the order *Eubacteriales* consists of gram-negative, nonsporulating rods. Among these, two large and two small groups are fairly well characterized by their morphology and physiology, namely the families *Pseudomonadaceae* (including *Vibrio* and *Spirillum*) and *Enterobacteriaceae* and the genera *Acetobacter* and *Azotobacter*. This leaves a very large group of bacteria unclassified. Among these are a few animal pathogens which proved to have several other properties in common: their outstanding character is their small size; then there is a certain similarity in their type of pathogenicity; and many of them are not readily cultivated on standard media.

These are the reasons why I proposed (1937) the grouping of these species in the family *Parvobacteriaceae*, so called because of the usually quite small size. This group has since been enlarged by the addition of similar bacteria which agree fairly well with the general properties of the group, but have some divergent characters through which the general agreement within the group has suffered. It is a question of expediency to decide whether to leave the family limited to its original species or to enlarge it in order to place apparently related species; whereby, however, some of the consistency of the group characterization must be sacrificed.

RT28. The Relationships between the Small, Gram-negative Bacteria, the Pleuropneumonia Group of Organisms, and Some Viruses. LOUIS DIENES-Massachusetts General Hospital, Boston, Mass.

The reproductive processes furnish important data for the classification of lower forms of life. A reproductive process different from binary fission has been observed in several bacterial species, and this process may help in the recognition of a relationship between the bacteria and some other organisms whose classification is now uncertain. A transition is apparent both in morphology and reproductive processes from the small, gram-negative bacteria through the pleuropneumonia group of organisms and such viruses as psittacosis and lymphogranuloma. The organisms of the latter two groups are tiny, gram-negative, bacillary forms which swell into round forms of variable size, reproducing the small forms again. A similar reproductive process has been observed in several species belonging to the *Enterobacteriaceae* and *Parvobacteriaceae*. It was observed only in freshly isolated strains and it appears to be related to pathogenicity. For example, in *Hemophilus influenzae* only strains isolated from severe infectious processes showed it. Binary fission seems to be supplemented with increasing parasitism by another type of reproductive process. The pleuropneumonia group and some viruses progress further in this direction than the bacteria, but the differences which separate these groups are quantitative only

and not qualitative. The peculiar reproductive process mentioned appears in variable forms in closely related bacterial strains and gives little help in the classification of bacteria at present.

RT29. The Genus *Proteus*. C. A. STUART, Brown University, Department of Bacteriology, Providence, R. I.

The genus *Proteus* when limited to four species, *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri*, represents a relatively compact group of organisms with closely related biochemical activities. All grow well on mediums designed to isolate pathogenic intestinal bacteria. Gas volumes produced in fermentable carbohydrates by the first three species are small under any conditions and old stock cultures are frequently anaerogenic. Most cultures of the fourth species are anaerogenic. Lactose is not attacked by any species and the number of sugars utilized by the group is somewhat limited. Swarming is characteristic of the genus but the facility of this reaction appears to decrease with the species in the order named above. Of the *Enterobacteriaceae* only *Proteus* gives strong evidence of rapid urease production in a strongly buffered medium. With respect to urease production (weakly buffered medium) *Proteus* appears to be more closely related to *Aerobacter* than other sections of the coliform group. Moreover, one species of *Proteus* frequently produces acetylmethylcarbinol.

Within each species there is marked continuity of antigens but, except for X strains, antigenically identical strains are exceptional. Overlapping antigens are encountered in the four species, and an occasional strain may possess minor antigens and a rare strain a major antigen in common with *Shigella*. Recent investigators have revealed at least one group of organisms which according to biochemical reactions occupy a position intermediate between *Proteus* and *Shigella*.

RT30. The Genus *Klebsiella*. ORREN D. CHAPMAN, Syracuse University College of Medicine, Syracuse, N. Y.

The organisms at present placed in the genus *Klebsiella* are gram-negative, nonmotile, plump, encapsulated rods with very variable fermentative and biochemical activities. They are usually isolated from the respiratory, gastrointestinal, and genitourinary tracts and certain skin lesions of man, but also may be found widely distributed in nature. Recent investigators agree concerning the difficulty in the identification of these organisms. When Julianelle's serological typing procedure is used as a criterion for identification, a highly variable group of gram-negative rods is found. Within a given serological type, lactose and non-lactose-fermenting strains may be found. The same variation can be demonstrated for practically all of the various biochemical tests commonly used in the study of the *Enterobacteriaceae*.

The classification of the family *Enterobacteriaceae* proposed by Borman, Stuart, and Wheeler in 1944 has much to recommend it and it may be possible to tentatively fit the organisms in *Klebsiella* into their genus *Paracolobactrum*. The difficulty is that many cultures recognized as *Klebsiella* ferment lactose rapidly.

It has been suggested by some that the *Klebsiella* may be considered as encapsulated varieties of *Escherichia coli*, *E. freundii*, *Aerobacter aerogenes*, and *A. cloacae*, but it is difficult to accept this view in the light of present knowledge. The final disposition of this group of microorganisms will depend upon extensive and detailed antigenic, pathogenic, and biochemical analysis.

RT31. Taxonomy and the Genus *Salmonella*. FREDERICK SMITH, McGill University, Department of Bacteriology and Immunology, Montreal, P. Q., Canada.

The history of the genus *Salmonella* during the last twenty years brings out two paramount facts: (1) that all the knowledge we possess of the biological significance and interrelation of the types of species within this genus we owe to antigenic analysis; (2) that there has been, and still is, considerable resistance to the acceptance of antigenic structure as a basis for taxonomy, even in a genus where such analysis has been the only profitable approach. Opposition to the use of antigenic structure has been based on the numerous fallacious serological relationships between biologically remote mammalian tissues, on the existence of identical antigens in bacteria of different families, and particularly on the rapidly mounting number of *Salmonella* types. At a minimal appraisal, epidemiological studies demand detailed *Salmonella* typing. Any attempt to compress the known types into a small number of species will nevertheless require the listing of the types within the species. A full and complete listing of all types is a more important consideration than is controversy over designation as types or species.

RT32. Spoilage in Processed Food. GLENN G. SLOCUM, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.

The shipment of decomposed food products is one of the major causes for action under the Federal Food, Drug, and Cosmetic Act, which declares a food to be adulterated under section 402(a)(3) "if it consists in whole or in part of a filthy, putrid, or decomposed substance." With modern advances in food technology there is little need for the distribution of processed foods which are undergoing or have undergone decomposition. Food decomposition is generally the result of carelessness and can be avoided by adequate inspection of raw materials, expeditious handling, proper processing, and suitable storage of the finished product.

The two broad classes of spoilage in processed foods with which the food law enforcement official is concerned are (1) the use of decomposed raw materials for the manufacture of foods in which the causes of spoilage cannot be determined by objective examination because they are removed or destroyed by subsequent processing and (2) active microbiological spoilage of food products, the causes for which can be determined by adequate laboratory investigation. There is need for fundamental research in the first class to determine the microorganisms responsible for spoilage, their sources, and methods for their elimination. Practically, decomposition of this type can be avoided as a cause for action under the Food, Drug, and Cosmetic Act by careful inspection of raw materials and expe-

ditious handling. The causes of active spoilage of food products are better known and can be eliminated largely by strict adherence to modern principles of food processing and handling.

RT33. Bacteriology of Frozen Foods. JAMES A. BERRY, Western Regional Research Laboratory, U. S. Department of Agriculture, Albany, Calif.

There is need for standardized methods in the bacteriological examination of frozen foods. Factors of importance are method of sampling, preparation of sample, medium, time and temperature of incubation. Tests for specific bacteria, as *Escherichia coli*, are of doubtful value, since this organism, like others, dies during freezing storage. A direct microscopic test would seem to be a better gauge of sanitary history than is the culture method. Bacteriological standards for frozen foods must be envisaged, but should be based on knowledge of reasonable procedures.

RT34. The Indications for Air Disinfection. STUART MUDD, University of Pennsylvania, Department of Bacteriology, Philadelphia 4, Pa.

Diseases transmitted principally by water, milk, and insect vectors have been brought under varying degrees of control through measures directed toward (a) interrupting transmission of the disease agents through the environment, and (b) actively immunizing the populations at risk. In the case of one great air-borne disease, epidemic influenza, notable progress in active immunization is currently reported. There is no assurance, however, that such vaccination could be relied upon against another, pandemic influenza. The banal air-borne infections, moreover, whose annual cost to American industry alone has been estimated at between a half billion and a billion dollars, are caused by a great variety of type-specific agents which do not readily lend themselves to active immunization procedures. Development and application of the arts of air disinfection, therefore, is one of the most challenging needs of preventive medicine.

Outdoor air is disinfected sufficiently for practical purposes by dilution and by solar irradiation. Disinfection of the air of confined spaces, in order to achieve practical values in public health, must meet certain critical specifications: (1) The rate of disinfection should be adequate materially to reduce the rate of person to person transfer of infectious agents. Certain ultraviolet installations have failed through too low intensity of radiation. (2) The susceptible population should be protected from air-borne disease agents wherever the risk of infection is material. Many installations have failed in practice because of exposures to infections elsewhere (e.g., in busses, trains, movies, etc.). (3) The disinfecting means should be entirely safe (cf. specifications of Council on Physical Therapy, J. Am. Med. Assoc. 122: 503).

RT35. The Use of Ultraviolet Radiation in Air Disinfection. ALEXANDER HOLLAENDER, National Institute of Health, Industrial Hygiene Research Laboratory, Bethesda 14, Md.

The radiation of 2537 Å is highly efficient in inactivating many disease agents. Since the introduction of sources of this radiation in inexpensive form, the use of ultraviolet for the control of air-borne infection has become a practical possibility. Although this possibility had been pointed out as long as fifteen years ago, experimental evidence indicating its usefulness in reducing air-borne infection is still limited. This paper will discuss its use in schools, crowded sleeping quarters, hospital operating rooms, and nurseries.

There are several things which have handicapped the development in this field: (1) The indiscriminate installation of ultraviolet sources in many places where they are of extremely little value and in many cases their use is entirely unpromising. (2) Installation of many of these lamps is done with little consideration for the safety of the occupants. The safeguards established by the Council of Physical Medicine are frequently ignored.

The author will illustrate these points from his own experiences in this field.

RT36. Disinfection of the Air by Oiling Floors and Bed Clothing. CLAYTON G. LOOSLI, MAJOR, M. C., The University of Chicago, Department of Medicine, Chicago, Ill.

Oiling floors and bedclothes is an effective and practical method of preventing the dispersal of dust-borne bacteria into the air from these secondary reservoirs. The amount and kind of oil employed in the treatment of floors depend on the type of floor or its covering and the frequency with which it is to be applied. It can be applied to soft- or hard-wood floors, varnished or unvarnished, waxed or unwaxed. It can be applied to linoleum or other composition floor coverings. For treatment of bedclothes an oil-in-water emulsion (T-13) has been developed by the Commission on Air-borne Infections, Army Epidemiological Board, Office of the Surgeon General, AUS. The T-13 base is composed by weight of 13 parts triton NE and 87 parts medicinal mineral oil. It is applied to the textiles in the form of a dilute oil-in-water emulsion in the same washwheel as the final rinse for a ten-minute period, following washing. The amount of oil base needed depends on whether the fabrics are woolen or cotton and whether they have been previously treated with oil. The T-13 oil-emulsion base is stable and the treatment inexpensive. Oil-treated fabrics are indistinguishable in appearance and texture from untreated ones, and are nonirritating and odorless. Oil-treated bedclothes with T-13 retain their ability to prevent dissemination of dust and bacteria into the air for long periods of time, particularly when used in conjunction with oil applied to the floors. Studies on the control of respiratory diseases by these methods are favorable, although inconclusive.

RT37. Studies by the Commission on Air-borne Infections on the Disinfection of Air by Glycol Vapors. THEODORE T. PUCK, University of Chicago, Department of Medicine, Chicago, Ill.

Study of the mechanism of chemical disinfection of air demonstrated that the view that collisions between bacteria and aerosol droplets of disinfectant are necessary for effective action was erroneous. Instead, the essential action con-

sists of concentration of free molecules of disinfectant in vapor form, upon bacterial droplets, until a lethal concentration accumulates. This concept led to the discovery of the efficacy of triethylene glycol, a substance potent in about 1/100 the concentration of propylene glycol. It was thus also possible to explain the influence exerted on this killing action by changing factors of relative humidity, temperature, and concentration of disinfectant in the air.

Laboratory studies tested whether inhalation of glycol vapors resulted in toxic manifestations. The data collected indicate that in amounts used for aerial disinfection these vapors do not produce any undesirable physiological effects. It was necessary to develop methods for detection and determination of minute amounts of triethylene glycol. A chemical method first devised has been succeeded by an instrument which measures the glycol vapor concentration and maintains a constant concentration at any desired level.

Field studies determined whether the bactericidal activity to tubercle bacilli under conditions could be achieved in hospital wards, etc. In the case of simultaneous hyperbacteremia in staphylococcal patients, the introduction of glycol vapor reduced the sensitivity of a hemolytic streptococci in the air by about 75 per cent, whereas in the laboratory complete sterilization of the air was attained. Analysis of this divergent behavior suggested that dried microorganisms in the air-borne dust and lint were not being killed as readily by the glycol. Later study in hospital wards revealed that the combination of glycol vapor plus dust-suppressive measures resulted in a 94 per cent reduction of streptococci in the air. Further field studies are being conducted to determine the epidemiological effects of glycol vaporization in preventing the spread of air-borne infection. In the laboratory the problems of the killing of the dried bacterial particle are being pursued, with the end in view of making dust-suppressive measures unnecessary.

RT38. Experience with the Practical Application of Triethylene Glycol Vapor for the Control of Air-borne Infection. EDWARD BIGG AND F. C. W. OLSON, Northwestern University Medical School, Department of Medicine, Chicago, Ill.

This presentation deals with a brief description of the types of apparatus developed for the generation and maintenance of bactericidal and virucidal triethylene glycol vapor concentration in occupied spaces. Several large-scale installations of these devices have been made in military cantonments, and pertinent bacteriologic and epidemiologic data has been collected. The results of these studies are summarized.

RT39. Serological Properties of Dextrans Formed from Sucrose by Certain Streptococci from Subacute Bacterial Endocarditis. EDWARD J. HEHRE AND JAMES M. NEILL, Cornell University Medical College, New York, N. Y.

The serological properties of dextrans formed from sucrose by certain streptococci from subacute bacterial endocarditis will be compared with those of dextrans formed by certain streptococci from human throats and by *Leuconostoc*

from plants. Points to be emphasized are the substrate specificity involved in the synthesis of the dextrans, the advantages of tests with a number of different antisera in determination of likenesses or differences among different dextrans, and the possible uses of dextran and levan formation in the differential description of nonhemolytic streptococci.

RT40. Immunochemical Studies on the *Shigella paradysenteriae* (Flexner). ELY PERLMAN, Mt. Sinai Hospital, New York, N. Y.

The purified somatic antigens of several types of *Shigella paradysenteriae* have been obtained by a variety of methods. These substances are lipocarbohydrate protein complexes which can be dissociated into a nontoxic polysaccharide hapten and toxic protein. They are potent pyrogens, induce hemorrhage in implanted mouse tumors, and produce necrotizing lesions in man as well as in experimental animals, giving rise to agglutinins, precipitins, mouse-protective and toxic antibodies. The author will illustrate.

RT41. The ^{infectious} Detoxification of Dysentery and Typhoid-soluble Antigens by Chemical Means. HENRY P. TREFFERS, Department of Immunology, Yale University School of Medicine, New Haven, Conn.

It is shown that acetylation of the soluble antigens of *Shigella dysenteriae* and *Eberthella typhosa* for varying lengths of time results in a series of products of 1/30 to 1/60 the toxicity of the native material, as judged by the effect on mouse weights. Other data are presented on the white cell responses of rabbits injected with the acetylated and unacetylated antigens.

The protective effect of the acetylated antigens against active infection of mice with these organisms is demonstrated, together with the lack of correlation of protection with agglutinin titers.

RT42. The Antigens of Epidemic and Murine Typhus *Rickettsiae* with Special Reference to Active Immunity in the Mouse. JAMES CRAIGIE, Connaught Laboratories, University of Toronto, Toronto, Can.

RT43. Products of Sonic Disruption of *Rickettsiae* and of Certain Bacterial Pathogens. LESLIE A. CHAMBERS. The Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa.

The chemical, physical, and antigenic properties of certain fractions obtained following sonic disruption of typhus rickettsiae will be reviewed in relation to the properties of related fractions of the same cells isolated following spontaneous and enzymatic lysis. Analogous properties of fractions of selected bacterial antigens following sonic disruption will be contrasted with the rickettsial fractions in relation to structural and metabolic differences between the two groups. The present status of sonic dispersion as a tool for antigenic fractionation will be discussed briefly with particular reference to suitable design of apparatus.

RT44. *Toxic Factors Obtained from Viruses.* HELEN P. JONES AND GEOFFREY RAKE, Squibb Institute for Medical Research, New Brunswick, N. J.

RT45. *Isolation and Properties of Factors Concerned in the Pathogenesis of Anthrax Infection.* DENNIS WATSON, Camp Detrick, Frederick, Md.

RT46. *The Cellular Transfer of Cutaneous Hypersensitivity.* MERRILL W. CHASE, Laboratories of The Rockefeller Institute for Medical Research, New York, N. Y.

Cells taken from sensitized guinea pigs and introduced into a normal guinea pig cause the recipient animal to acquire the specific hypersensitivity of the animals furnishing the cells. By this means, the "delayed" or "contact-dermatitis" type of hypersensitivity to drugs (picryl chloride, 2:4 dinitrochlorobenzene, o-chlorobenzoyl chloride) and the delayed type of hypersensitivity to tuberculin have been transferred. Animals developing intense cutaneous hypersensitivity to picryl chloride have not, however, become anaphylactically sensitive. The transferred sensitivity arises after a latent period of one to two days and has a limited duration, receding sharply four or five days after it is first seen. The cells may be obtained from exudates, splenic pulp, or lymph nodes. Transfers have not been successful with cells damaged by freezing or heating.

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LITMOCIDIN, A NEW ANTIBIOTIC SUBSTANCE PRODUCED BY PROACTINOMYCES CYANEUS

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In a search for new antibiotic substances a soil actinomycete was isolated by us from a sample of south Russian soil that produces a powerful antibiotic substance of a new type. In this article a description of this organism is given, together with some details concerning the production of the antibiotic substance and its action upon pathogenic bacteria. A subsequent paper by M. G. Brazhnikova treats of the isolation and purification of litmocidin and outlines its chemical nature.

The antagonist. The strain of actinomycete isolated by us is close to *Actinococcus cyaneus*, isolated by Beijerinck from garden soil in Holland in 1913. It forms well-developed mycelium which later disintegrates into single cells. Colonies produce deep-blue water-soluble pigment, which becomes red at an acid reaction, and blue at an alkaline reaction. A similar litmuslike pigment was recorded by Beijerinck.

However, the conditions for the production of a litmuslike pigment by our microbe and by the *Actinococcus* of Beijerinck are very different. The latter readily produced pigment upon a mineral medium containing glucose and ammonium nitrate. Upon this medium our microbe is unable to produce a litmuslike pigment. The conditions for pigment production by our microbe are outlined in table 1. It is clear that either peptone or tryptone (still better both of them) is required for pigment production in our strain.

In view of important differences in pigment production between our microbe and the *Actinococcus* of Beijerinck, and because of the strong antibiotic action of the litmuslike pigment produced by our microbe (nothing like this is reported by Beijerinck), it is proposed to designate the strain as a new variety, var. *antibioticus*. According to modern classification (Krasilnikov, 1945) the generic name *Actinococcus* should be replaced by *Proactinomyces*, and the complete name of the organism should be *Proactinomyces cyaneus-antibioticus*.

It is to be pointed out that the production of a litmuslike pigment occurs in different groups of actinomycetes, and has been particularly well studied in *Actinomyces coelicolor*. As Kriss (1936) has shown, the pigment of the latter species belongs to the anthocyanines. It is also remarkable that the litmuslike pigment of *A. coelicolor* is devoid of antibiotic action (Krasilnikov, 1945). The anthocyanine pigments of plants are also devoid of antibiotic action, according to our experience.

In the light of all this evidence it appears probable that the antibiotic, litmuslike pigment observed by us represents some specific modification of the common litmuslike pigment. As will be shown in the subsequent paper, the antibiotic

substance recorded by us represents some derivative of anthocyanidine and evidently differs from the familiar anthocyanine pigments of plants. Inasmuch as the new antibiotic substance represents a litmus pigment with antibacterial action, it is proposed to designate it as *litmocidin*.

Formation of litmocidin. To obtain litmocidin, *P. cyaneus-antibioticus* is grown upon a nutritive agar of the following composition: peptone 0.5 per cent, tryptone 0.3 per cent, glucose 1 per cent, NaCl 0.5 per cent, FeSO₄ 0.001 per cent, agar 2 per cent, tap water. While growing upon this medium *P. cyaneus-antibioticus* strongly inhibits the growth of staphylococci. The antagonistic organism grows very rapidly, and the litmocidin produced by it reaches maximal concentration in the nutritive agar 48 to 72 hours after inoculation at 28 C. The

TABLE 1

Growth and pigment production by Proactinomyces cyaneus-antibioticus upon various media

COMPOSITION OF NUTRITIVE AGAR	GROWTH	PIGMENT PRODUCTION
1. Peptone, glucose, mineral salts.	Good	Observed (++)
2. Tryptone, glucose, mineral salts. . . .	Good	Observed (++)
3. Peptone, tryptone, glucose, mineral salts.	Good	Very strong (++++)
4. Meat extract, glucose, mineral salts.	Good	No pigment
5. Pea extract.	Good	No pigment
6. Yeast autolysate	Good	No pigment
7. Asparagine, glucose, mineral salts . . .	Good	No pigment
8. Ammonium nitrate, glucose, mineral salts.	Good	No pigment

TABLE 2

The inhibition of growth of Staphylococcus aureus in broth by the watery extract of a 48-hour-old agar culture of P. cyaneus-antibioticus

DILUTION OF EXTRACT	1:50	1:100	1:250	1:500	1:750	1:1,000	1:1,500
Growth of staphylococci	—	—	—	—	—	—	+

inhibitory action upon staphylococci of the watery extract of an agar culture is shown in table 2.

Strain variation. In the course of subsequent cultivation the strain of *P. cyaneus-antibioticus* isolated by us produced inactive variants differing from the basic active strain:

- | Active strain | Inactive variant |
|--|--|
| 1. <i>Antibiotic activity.</i> Abundantly produces litmocidin in agar cultures. | 1. No litmocidin or only traces of litmocidin are formed. |
| 2. <i>Growth.</i> No sporulating aerial mycelium. Growth characteristic of <i>Proactinomyces</i> . | 2. Colonies are covered by a thin layer of grayish-white aerial mycelium. |
| 3. <i>Variation.</i> Nonsporulating strain gives rise to sporulating variants. | 3. No reconversion of sporogenous variants into original asporogenous form has so far been recorded. |

The situation observed by us is in a sense the reverse of that recorded by Schatz and Waksman (1945) in *Actinomyces griseus*. For the successful production of litmocidin the cultures of *P. cyaneus-antibioticus* are regularly plated upon nutritive agar; active nonsporulating variants are selected and kept in pure culture.

Unit of activity of litmocidin is that quantity of the substance which is just sufficient to inhibit completely the growth of *Staphylococcus aureus* in 1 ml of broth. One ml of a two-day-old agar culture of *P. cyaneus-antibioticus* contains 2,000 units of litmocidin.

TABLE 3

The bacteriostatic action of litmocidin upon various bacteria in nutritive broth

BACTERIA	DILUTION OF LITMOCIDIN COMPLETELY INHIBITING GROWTH
<i>Staphylococcus aureus</i> 1418.....	1:4,000,000
<i>Staphylococcus aureus</i> M.....	1:4,000,000
<i>Staphylococcus aureus</i> 75a.....	1:2,000,000
<i>Staphylococcus aureus</i> 1623.....	1:2,000,000
<i>Streptococcus hemolyticus</i> 8.....	1:1,000,000
<i>Streptococcus hemolyticus</i> T.....	1:2,000,000
<i>Streptococcus hemolyticus</i> D.....	1:2,000,000
<i>Streptococcus hemolyticus</i> 5.....	1: 400,000
<i>Streptococcus hemolyticus</i> 1972.....	1: 500,000
<i>Streptococcus hemolyticus</i> 11.....	1:2,000,000
<i>Streptococcus hemolyticus</i> 13.....	1:1,000,000
<i>Streptococcus viridans</i>	1: 500,000
<i>Mycobacterium tuberculosis</i>	Very strong action
<i>Vibrio comma</i> 23.....	1:1,000,000
<i>Vibrio comma</i> 15.....	1:1,000,000
<i>Vibrio comma</i> 72.....	1:1,000,000
<i>Vibrio comma</i> 1606.....	1:1,000,000
<i>Vibrio comma</i> 771.....	1: 500,000
<i>Vibrio comma</i> 99.....	1:2,000,000
<i>Vibrio comma</i> U.....	1:2,000,000
<i>Shigella dysenteriae</i> (Shiga).....	1: 10,000
<i>Shigella dysenteriae</i> (Flexner).....	1: 10,000
<i>Eberthella typhosa</i>	1:1,000
<i>Salmonella paratyphi</i> A.....	1:1,000
<i>Salmonella paratyphi</i> B.....	1:1,000
<i>Escherichia coli</i>	1:1,000

As will be shown in the subsequent paper, the active principle of litmocidin is now isolated and purified, and it is available as a dry powder. The activity of this powder is 4,000 units per mg.

Action of litmocidin upon bacteria. The experiments here reported were made with a purified preparation of litmocidin. Usually we made a 0.2 per cent solution of litmocidin in water, which preparation possesses an acid reaction and a red coloration. With a little alkali the reaction was made neutral, and the solution acquired a violet color. The bacteriostatic action of litmocidin upon various bacteria is shown in table 3.

It is clear that litmocidin possesses selective action. It strongly inhibits staphylococci, streptococci, tubercle bacilli, and *Vibrio comma*, whereas its action upon dysentery bacilli is moderate, and upon typhoid and colon bacilli there is practically no action at all. For most strains of staphylococci, streptococci, and *Vibrio comma* the action of litmocidin is not only bacteriostatic, but also bactericidal. It is also remarkable that the antibacterial action of litmocidin is in no way inhibited by the addition of 10 per cent to 30 per cent of human or horse serum to the nutritive medium.

Toxicity of litmocidin. Neutral 0.2 per cent to 0.4 per cent solutions of litmocidin in physiological saline were given intraperitoneally to white mice 20 grams in weight. The observations continued for 5 days, and the corresponding data are presented in table 4. This table shows that a single intraperitoneal administration is not very toxic for mice.

TABLE 4
Toxicity of litmocidin for mice

UNITS OF ACTIVITY PER 20-g MOUSE	NUMBER OF MICE	PERCENTAGE OF MORTALITY
1,000	20	0
1,500	20	0
2,000	15	0
2,500	15	0
3,000	15	0
4,000	25	52
5,000	25	48
10,000	20	100

Chemotherapeutic activity. White mice were infected intravenously with a virulent strain of *Staphylococcus aureus* 1418, which is inhibited by litmocidin *in vitro* in a dilution of 1:4,000,000. Watery solutions of litmocidin were injected subcutaneously into infected mice in amounts of 50 to 500 units, 1 to 3 times per day, for 3 consecutive days. These experiments have shown that litmocidin does not possess chemotherapeutic action on staphylococcal septicemia in mice. The author is indebted to Professor A. Pines for making the chemotherapeutic trial of litmocidin.

SUMMARY

A new variety of *Proactinomyces cyaneus* has been isolated from a sample of south Russian soil and is described as *P. cyaneus-antibioticus*. It produces an antibiotic substance of a new type, representing a derivative of anthocyanidine. This antibiotic is designated as litmocidin. It reveals strong bacteriostatic action upon staphylococci, streptococci, *Vibrio comma*, and tubercle bacilli, and this action is not inhibited by blood serum. Litmocidin is devoid of chemotherapeutic action on septicemia in mice caused by a strain of staphylococcus very susceptible to litmocidin *in vitro*.

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THE ISOLATION, PURIFICATION, AND PROPERTIES OF LITMOCIDIN

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It was shown by Gause (1946) that *Proactinomyces cyaneus-antibioticus* produces an antibiotic substance of a new type, designated as litmocidin. The present article describes the isolation and purification of litmocidin and outlines some of its properties.

ISOLATION OF LITMOCIDIN

Extraction of the active principle from agar culture. Observations have shown that watery extracts of agar cultures of *P. cyaneus-antibioticus* contain a blue pigment and are very active in suppressing the growth of staphylococci. This pigment possesses indicator properties: it is red at an acid reaction, violet at neutrality, and blue at an alkaline reaction. It was also found that this pigment cannot be extracted from agar cultures by ethanol, ether, or acetone at a neutral reaction. Extracts made with these organic solvents after evaporation yield a colorless residue, which possesses no antibiotic activity. However, when organic solvents are acidified to a pH of 3.0, the pigment readily passes from agar into the solvent in the following order: ethanol, acetone, ether, amyl acetate. The evaporation of organic solvents leaves a red residue, which possesses strong antibacterial action. It was hence concluded that antibacterial activity is associated with the pigment, which is soluble in organic solvents at an acid reaction.

Isolation of the active principle from watery extracts. The antibacterial pigment readily passes from watery extracts of agar cultures into ether at a pH of 3 to 4. At a neutral reaction it is insoluble in ether, and hence differs in this respect from actinomycin studied by Waksman and Woodruff (see Waksman, 1945) and from mycetin (Fainschmidt and Koreniako, 1944). However, when a watery extract is acidified to a pH of 3 to 4 an abundant black sediment is immediately formed. It was found that this sediment consists of inactive protein and active pigment adsorbed upon it. These two components are easily separated in the following way: The sediment is filtered, washed, and dissolved in alkaline water. When the water is acidified, the sediment comes out again, leaving some of the pigment in the watery solution. This sedimentation was repeated 12 times and finally yielded pure protein without antibacterial action.

It was further recorded that the watery extract of an agar culture contains two pigments. One of these (litmocidin) is extracted by ether at an acid reaction, changes from red to blue with a rise in pH, and possesses antibiotic action. Another pigment, present in small amount, is insoluble in ether at an acid reaction, changes from orange to green with a rise in pH, and is inactive against bacteria.

Preparation of dry litmocidin. It was found possible to prepare litmocidin in the following way: To a watery extract of an agar culture 1 per cent of charcoal is added, and the mixture is acidified to pH 3.5. In this procedure the pigment is completely adsorbed on the charcoal, and the liquid becomes colorless. The charcoal is filtered, and further repeatedly extracted by acetone at an acid reaction. The acetone is evaporated *in vacuo* to dryness, and the residue is dissolved in a small amount of ethanol. To four parts of ethanol one part of water is added, and the pigment is immediately sedimented. The sediment is centrifuged, dried *in vacuo*, and kept in powdered form.

Purification of litmocidin. Dry litmocidin can be further purified in the following way: It is dissolved in ethanol, and a few drops of muriatic acid are added to it. The ethanol solution is then mixed with ether, and water is added to this mixture until a watery layer clearly separates. Litmocidin passes into the ether, and impurities are left in the watery phase. This procedure is repeated several times. Finally, the ether is evaporated, the dry residue is dissolved in ethanol, and pure litmocidin is sedimented by water as described above.

PROPERTIES OF LITMOCIDIN

Acid and alkaline forms of litmocidin. The preparation of the acid (red) form of litmocidin has already been described. It is slightly soluble in water at acid reactions and soluble in ethanol and acetone. At a neutral reaction its solubility in water is greatly increased. When the acid (red) form of litmocidin is dissolved in ethanol and neutralized by alkali, and the ethanol evaporated *in vacuo*, the blue (alkaline) form of the pigment is obtained. This form is readily soluble in water. Both forms inhibit the growth of *Staphylococcus aureus* in nutrient broth in a dilution of 1:4,000,000.

Stability of litmocidin. Dry acid litmocidin is entirely stable. Boiling of 0.01 per cent watery solutions of litmocidin for 30 minutes under different conditions shows that it is stable at acid reactions, but at alkaline reactions the activity and coloration rapidly disappear.

It is possible to undertake a differential destruction of the coloration and the activity of litmocidin. Strong alkalies at room temperature destroy the activity but do not affect the color of litmocidin. On the other hand, the heating of litmocidin with 20 per cent HCl for 10 hours at 80 to 90 C affects the coloration but does not interfere with the antibiotic action of litmocidin.

Relation of litmocidin to anthocyanins. Litmocidin has much in common with the anthocyanin pigments of plants, and a comparison of some of their properties is presented below.

Properties of anthocyanins (Onslow, 1925)

1. Green or blue at alkaline reactions.
2. Red at acid reactions.
3. Green or blue sediment with lead acetate.

Properties of litmocidin

1. Blue at alkaline reactions.
2. Red at acid reactions.
3. Blue sediment with lead acetate.

Properties of anthocyans

4. Anthocyanidines (pigments of anthocyans) are completely extracted from water by amyl alcohol at an acid reaction.
5. Acid solutions of anthocyans become colorless when treated with zinc dust (i.e., by nascent hydrogen). On exposure to air the color returns.
6. Decolorized by addition of sodium bisulfite. The color returns on addition of strong acid.

Properties of litmocidin

4. Litmocidin is completely extracted from water by amyl alcohol at acid reaction. When reaction is made alkaline, it passes into water again and acquires a blue color.
5. Acid solutions of litmocidin are decolorized by zinc dust. On exposure to air the color returns.
6. Decolorized by bisulfite, but the color is not restored by strong acid.

It is clear that the pigment properties of litmocidin have much in common with the anthocyanidines. But whereas the anthocyanidine pigments of plants are combined with carbohydrates, it was found that litmocidin does not contain any carbohydrate in its molecule.

Repeated purification of litmocidin does not increase further its antibacterial action. Hence it is clear that we are dealing here with an individual body. Litmocidin also possesses a constant melting point. It melts (with decomposition) at 144 to 146 C.

SUMMARY

Litmocidin, a new antibiotic substance produced by *Proactinomyces cyaneus-antibioticus*, has been isolated and purified. It possesses a constant melting point (144 to 146 C). Litmocidin is a pigment, and it has much in common with the anthocyanin pigments of plants. Litmocidin can be obtained in two forms: acid (red) and alkaline (blue), possessing different solubilities in water. Both forms inhibit the growth of *Staphylococcus aureus* in a dilution of 1:4,000,000.

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THE MECHANISM OF BACTERIAL AND FUNGUS GROWTH INHIBITION BY 2-METHYL-1,4-NAPHTHOQUINONE

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Early work including that of Browne (1906), Thalheimer and Palmer (1911), Cooper (1912), and Morgan and Cooper (1921, 1924), suggested, demonstrated, and confirmed the fact that certain quinones are bactericidal. Interference with this property by serum and peptones precluded their use as germicides in man (Morgan and Cooper, 1924). Interest in quinones as antibacterial agents, however, has been stimulated somewhat in recent years by the discovery by Oxford and Raistrick (1942) and by Waksman and Woodruff (1942) of the quinone structure in certain antibiotics derived from molds. Fosdick, Fancher, and Calandra (1942) found that 2-methyl-1,4-naphthoquinone inhibited acid formation from glucose in saliva without effect on bacterial growth in the concentration employed (1 mg per 100 ml). Armstrong and his co-workers (1943) claimed that this effect was due to the quinone structure and noted *in vitro* inhibition of certain strains of streptococcus, staphylococcus, and pneumococcus by several quinones, including 2-methyl-1,4-naphthoquinone. They stated that none of the quinones tested in a concentration as high as 29×10^{-6} mols per 100 ml inhibited the growth of *Escherichia coli*. Observations of fungus-inhibitory properties of 2-methyl-1,4-naphthoquinone by Hyman and Sheehy of this company (unpublished) have received confirmation in a recent report by Gonzalez (1945).

Synthetic 2-methyl-1,4-naphthoquinone as tested in this laboratory has also been found bacteriostatic and bactericidal for both gram-negative and gram-positive bacteria. In the course of studies made with *Escherichia coli* it was noted that minimal effective antibacterial concentrations were lower in a chemically defined medium than in the usual peptone broth or agar (0.00125 per cent to 0.0025 per cent instead of 0.005 per cent to 0.02 per cent); also that in freshly prepared Brewer's medium antibacterial effects were nullified, although in the same medium prepared some three months previous to use and thoroughly shaken to insure oxygenation, bacteriostatic and bactericidal end points were similar to those obtained in other peptone media. The effect of the Brewer medium did not appear to be due to its anaerobic properties, since tests incubated in an anaerobic jar had the same end points as those incubated in air. The following work was undertaken to explain this behavior (Colwell and McCall, 1945).

METHODS

Quinones employed included 2-methyl-1,4-naphthoquinone, the water-soluble sodium bisulfite addition product of 2-methyl-1,4-naphthoquinone, 2-methyl-3-chloro-1,4-naphthoquinone, 2,6-dimethyl-1,4-naphthoquinone, and 2-methyl-3-methoxy-1,4-naphthoquinone, all pure synthetic compounds prepared in the

chemical research laboratories of this company. Substances tested for antagonism of antibacterial properties of these quinones were (a) methylene blue and sodium thioglycolate (two ingredients of Brewer's medium not present in the usual peptone media), (b) a group of reducing agents, some of which contained sulfur, (c) a group of other compounds containing the —SH radical, and (d) *l*(—)cystine, with the S—S bond instead of the —SH group. These compounds were made up in a sterile aqueous solution, adjusting the pH when necessary.

Antibacterial tests. MacLeod's "synthetic" medium (1940), Difco nutrient broth buffered with disodium phosphate, and Difco tryptose phosphate broth, dispensed in accurately measured amounts in 8" by 1" test tubes, were the media used. Test organisms were stock laboratory strains of *Escherichia coli* and *Staphylococcus aureus* 313.¹ Cultures of *E. coli* used in "synthetic" medium had been transplanted at least 6 times in asparagine medium to eliminate possible traces of peptone, and a stock culture was then maintained on asparagine medium containing glycerol instead of glucose to lessen dissociation (MacLeod, 1940).

Tests were made as follows: Solutions of quinones were prepared aseptically in acetone or water, depending on their solubilities, in 25 times the greatest final concentration desired in tests. Serial dilutions were made by halves and were added in 1-ml amounts to 24 ml of sterilized medium (to 23 ml if a substance to be tested for antagonism of antibacterial action was also to be added). Standard dilutions of substances to be tested for antagonism of antibacterial action were added in 1-ml amounts to each tube of a series containing varying concentrations of quinone. Acetone and water controls were included in each series. The inoculum of bacteria was 0.1 ml of a 1:4 dilution of a 24-hour culture in appropriate medium (approximately 20 to 30 million bacteria, or 800,000 to 1 million per ml of culture medium). Incubation was at 37 C except when the volatile ethyl mercaptan was used; then both mercaptan and control series were incubated at 30 C. Readings for bacteriostasis were made daily for 4 to 5 days by visual observations of turbidity. Daily subcultures were made by streaking each culture on an agar plate to determine whether bacteria were killed or merely kept from growing.

Fungicidal tests. Test molds were *Aspergillus niger* strain 6277 (A.T.C.C.) and mold F.P.L. no. 517 (Richards, 1937). Tests were made on malt extract agar using the method of Schmitz and others (1930) and were incubated at 30 C. Substances were tested for antagonism of antifungal activity in a manner similar to that used in antibacterial tests. After a 14-day period of observation for fungistasis, inocula which had failed to grow were transplanted to fresh medium without fungicide and incubated to determine whether molds had been killed.

Nitroprusside tests for sulfhydryl groups were made on mixtures of equal parts of alcoholic solutions of quinones and aqueous solutions of (a) *l*(+)cysteine hydrochloride and (b) sodium thioglycolate, at intervals, after incubation of the mixtures at 37 C for as long as 20 hours.

¹ Obtained through the courtesies of Dr. A. W. Walker and Dr. G. P. Youmans, respectively, of the Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois.

RESULTS

Antibacterial tests. Repeated tests in the same medium gave consistent results, daily readings varying by not more than one dilution and the end result, after readings no longer changed, being the same, with the one exception noted below. Typical results of daily reading are given in table 1. Interference by peptone is readily detected by comparing the end points of the same quinone for the same test organism in different media.

The sodium bisulfite addition product of 2-methyl-1,4-naphthoquinone was decidedly inferior as an antibacterial substance to the quinone itself; moreover, different samples of crystals of this product often gave different end points. An explanation for this variation was suggested as the work progressed.

Staphylococcus aureus was inhibited by slightly lower dilutions of 2-methyl-1,4-naphthoquinone than was *E. coli*, but with 2-methyl-3-chloro-1,4-naphthoquinone the reverse was true, *E. coli* being the more sensitive of the two organisms. 2,6-Dimethyl-1,4-naphthoquinone was a poor antibacterial substance.

Methylene blue in the concentration employed in Brewer's medium did not affect the end points of 2-methyl-1,4-naphthoquinone for *E. coli* in the chemically defined medium. Sodium thioglycolate, however, when in excess on a molar basis, prevented the antibacterial activity of this quinone on both *S. aureus* and *E. coli*. Thioglycolate prepared by sodium carbonate neutralization of an old laboratory stock of thioglycolic acid appeared to be more pure than Eastman's sodium thioglycolate.² This is confirmation of the contention of Foley and Maren (1944) that commercial thioglycolate as now supplied is grossly impure.³

Quantitative relationships between concentrations of quinone and thioglycolate prepared by neutralizing thioglycolic acid are illustrated in table 2. Halving the concentration of thioglycolate in the test permitted the antibacterial activity of half the concentration of quinone; that is, an excess of thioglycolate on a molar basis was essential for antagonism of antibacterial activity.

Sodium thiosulfate and potassium formate in great excess over the quinone did not interfere with the latter's antibacterial activity (table 2). Sodium bisulfite and sodium hydrosulfite, however, suppressed the antibacterial activity of 2-methyl-1,4-naphthoquinone, as did ethyl mercaptan and *l*(+)-cysteine hydrochloride, while *l*(-)-cystine, with the S—S bond instead of the —SH group, had only a slight antagonistic action, end points with this compound being almost within the limits of error of the test (table 3).

Suppression of the antistaphylococcus activity of 2-methyl-1,4-naphthoquinone in buffered nutrient broth was similar to the suppression of the anticoli activity in asparagine medium except that cysteine was less completely effective

² Thioglycolate prepared by neutralizing thioglycolic acid, in a concentration of 2×10^{-3} M, inactivated 2-methyl-1,4-naphthoquinone in a concentration of 1.16×10^{-3} M, but the inactivation of the same amount of quinone by Eastman's thioglycolate required the latter in a concentration of 50 to 100×10^{-3} M, a concentration of 20×10^{-3} M being ineffective.

³ CP sodium thioglycolate recently received from the Wallace Laboratories has been found to suppress antibacterial activity of 2-methyl-1,4-naphthoquinone in concentrations similar to those of the neutralized thioglycolic acid.

TABLE 1
The antibacterial effect of quinones

QUINONE	MEDIUM	MINIMUM EFFECTIVE MOLAR CONCENTRATIONS*									
		Bacteriostatic					Bactericidal				
		24 hr	48 hr	72 hr	96 hr	5 days	24 hr	48 hr	72 hr	96 hr	5 days
Test organism: <i>E. coli</i>											
2-me-1,4-n.q.	Synth.	3.6	7.26	7.26	7.26	7.26	29	14.5	14.5	7.26	7.26
	B.N.B.	29.0	29.0	58.0	58.0		116	116.0	58.0	58.0	
	T.P.B.	116.0	116.0	116.0	116.0		116	116.0	116.0	116.0	
NaHSO ₃ add. prod. of 2-me-1,4-n.q.	Synth.	Variable					Variable				
	B.N.B.	>145.0	>145.0				>145.0	>145.0			
2-me-3-Cl-1,4-n.q.	Synth.	3.0	3.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
	Synth.	6.18	6.18	12.3	12.3	24.7	>99.0	>99.0	49.5	12.3	
2-me-3-methoxy-1,4-n.q.	B.N.B.	24.7	49.5	99.0	99.0	99.0	>198.0	>198.0	198.0	198.0	198.0
	T.P.B.	99.0	>198.0	>198.0	>198.0	>198.0	>198.0	>198.0	>198.0	>198.0	
Test organism: <i>Staphylococcus aureus</i> 313											
2-me-1,4-n.q.	B.N.B.	7.26	14.5	29.0	29.0	29.0	58.0	29.0	29.0	29.0	
	T.P.B.	14.5	29.0	29.0	29.0	29.0	116.0	116.0	58.0	58.0	58.0
2-me-3Cl-1,4-n.q.	B.N.B.	12.0	24.0	24.0	48.0		193.0	48.0	48.0	48.0	
	B.N.B.	12.3	24.7	24.7	24.7		>198.0	>198.0	>198.0	>198.0	198.0
2-me-3-methoxy-1,4-n.q.											

Synth. = asparagine medium.

B.N.B. = nutrient broth buffered with 0.25 per cent Na₂HPO₄.

T.P.B. = tryptose phosphate broth.

* All figures should be multiplied by 10⁻³.

TABLE 2
The effect of reducing agents on the antibacterial action of quinones

RED. AGENT	M CONC.*	MINIMUM EFFECTIVE MOLAR CONCENTRATIONS OF QUINONES*									
		Bacteriostatic					Bactericidal				
		24 hr	48 hr	72 hr	96 hr	5 days	24 hr	48 hr	72 hr	96 hr	5 days
		Test organism: <i>E. coli</i> I Medium: asparagine-synthetic Quinone: 2-methyl-1,4-naphthoquinone									
O		3.6	7.26	7.26	7.26	7.26	29.0	14.5	14.5	7.26	7.26
Na-thiosulfate	1,000	3.6	7.26	7.26	14.5		29.0	14.5	14.5	14.5	
K-formate	4,760	3.6	7.26	7.26	7.26		14.5	14.5	14.5	14.5	
Na-hydrosulfite	2,270	116.0	232.0	232.0	232.0		>232.0	232.0	232.0	232.0	
Na-thioglycolate	200	116.0	232.0	232.0	232.0	232.0	232.0	232.0	232.0	232.0	232.0
	100	116.0	116.0	116.0	116.0	116.0	232.0	116.0	116.0	116.0	116.0
	50	58.0	58.0	58.0	58.0	58.0	232.0	58.0	58.0	58.0	
	1,000	>232.0	>232.0				>232.0	>232.0			
Na-bisulfite	333	116.0	116.0	116.0	232.0	232.0	>232.0	232.0	232.0	232.0	
	166	58.0	58.0	58.0	116.0	116.0	232.0	116.0	116.0	116.0	116.0
	83	29.0	58.0	58.0	58.0	58.0	232.0	58.0	58.0	58.0	58.0
Quinone: 2-methyl-3-methoxy-1,4-naphthoquinone											
O		6.18	6.18**	12.3**	12.3**		>198.0	99.0	49.5	12.3	
Na-bisulfite	333	6.18	12.3**	24.7**	24.7**		>198.0	>198.0	98.0	24.7	
Test organism: <i>Staphylococcus aureus</i> 313 Medium: buffered nutrient broth Quinone: 2-methyl-1,4-naphthoquinone											
O		7.26	29.0	29.0	29.0	29.0	58.0	29.0	29.0	29.0	
Na-bisulfite	333	116.0	232.0	232.0	232.0	232.0	>232.0	232.0	232.0	232.0	
Quinone: 2-methyl-3-methoxy-1,4-naphthoquinone											
O		12.3	24.7**	24.7**	24.7**		>198.0	>198.0	>198.0	198.0	
Na-bisulfite	333	24.7	24.7**	24.7**	49.5**	49.5**	>198.0	>198.0	198.0	99.0	

* All figures should be multiplied by 10⁻⁴.

** Quinone has changed color from yellow to red, probably indicating formation of 2-methyl-3-hydroxy-1,4-naphthoquinone.

Test organism: *Staphylococcus aureus* 313
Medium: buffered nutrient broth.—Quinone: 2-methyl-1,4-naphthoquinone

O		Quinone: 2-methyl-3-chloro-1,4-naphthoquinone							
		7.26	29.0	29.0	29.0	116.0	29.0	29.0	29.0
I(+)-cysteine HCl	333	29.0	58.0	58.0	58.0	116.0	58.0	58.0	58.0
Na-thioglycolate	333	232.0	>232.0	>232.0	>232.0	>232.0	>232.0	>232.0	>232.0
I(-)-cystine**	333	14.5	29.0	29.0	29.0	29.0	29.0	29.0	29.0
O		Quinone: 2-methyl-3-methoxy-1,4-naphthoquinone							
		12.0	24.0	24.0	48.0	193.0	48.0	48.0	48.0
I(+)-cysteine HCl	333	96.0	193.0	193.0	193.0	>193.0	193.0	193.0	193.0
	166	96.0	96.0	193.0	193.0	193.0	193.0	193.0	193.0
Na-thioglycolate	83	48.0	48.0	48.0	48.0	193.0	96.0	96.0	96.0
	333	96.0	>96.0	>96.0	>96.0	>96.0	>96.0	>96.0	>96.0
I(-)-cystine**	333	12.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0
O		Quinone: 2-methyl-3-methoxy-1,4-naphthoquinone							
		12.3	24.7	24.7†	24.7†	>198.0	>198.0	>198.0	198.0
I(+)-cysteine HCl	333	12.3	24.7	24.7†	49.5†	>198.0	>198.0	198.0	99.0
Na-thioglycolate	333	12.3	24.7	49.5†	49.5†	>99.0	>99.0	>99.0	>99.0

* All figures should be multiplied by 10⁻⁴.

** S—S bond instead of —SH group.

† Color changed from yellow to red, probably indicating formation of 2-methyl-3-hydroxy-1,4-naphthoquinone.

in the former tests. Whether this was due to the medium or the test organism was not determined. Excesses of cysteine and bisulfite were essential, as with sodium thioglycolate (tables 2 and 3). In the presence of equimolar concentrations of bisulfite, however, 2-methyl-1,4-naphthoquinone had about the same end points as in control tests without bisulfite.

Thioglycolate, cysteine, and bisulfite excesses also prevented the antibacterial activity of 2-methyl-3-chloro-1,4-naphthoquinone on *S. aureus* and *E. coli*. When thioglycolate, cysteine, and bisulfite were used with 2-methyl-3-methoxy-1,4-naphthoquinone, however, there was little effect on end points, at least until

TABLE 4
The fungicidal activity of quinones

QUINONE	ANTAGONIST	M CONC*	MOLD F.P.L. 517	ASPERGILLUS NIGER 6277		
			Minimum effective molar concentrations of quinones*			
			Fungi- static	Fungi- cidal	Fungi- static	Fungi- cidal
2-Methyl-1,4-naphtho- quinone	O		58	58	116	116
	Na-thioglycolate	250	232	232	> 232	> 232
		125	232	232	232	232
		62.5	116	116	232	232
	l(+)-cysteine HCl	416	> 232	> 232	> 232	> 232
	Na-bisulfite	300	> 232	> 232	232	232
	l(-)-cystine	416	58	58	116	116
2-Methyl-3-methoxy- 1,4-naphthoquinone	O		24.75	24.75	24.75	> 198
	Na-thioglycolate (East- man)	10,000	24.75	24.75	24.75	> 198
	l(+)-cysteine HCl	416	24.75	24.75	24.75	> 198
	Na-bisulfite	333	24.75	24.75	24.75	> 198
	l(-)-cystine	416	49.5	49.5	24.75	> 198

* All figures should be multiplied by 10^{-5} .

the third or fourth day of incubation. After this length of time 2-methyl-3-methoxy-1,4-naphthoquinone had undergone a color change, from yellow to red. Since it is known that the methoxy quinone is unstable in alkaline medium, tending to form 2-methyl-3-hydroxy-1,4-naphthoquinone, it is improbable that after prolonged incubation periods we were dealing with the pure methoxy quinone.

Fungicidal tests. 2-Methyl-1,4-naphthoquinone under the conditions of these experiments killed mold 517 in a dilution of 58×10^{-5} M and *Aspergillus niger* in a dilution of 116×10^{-5} M. Lower concentrations retarded but did not completely inhibit growth. Sodium thioglycolate, cysteine, and sodium bisulfite in excesses over the quinone prevented killing of both test molds (table 4).

2-Methyl-3-methoxy-1,4-naphthoquinone, presumably stable in the acid mold medium, was fungicidal for mold 517 in a concentration of 24.75×10^{-5} M, but was only fungistatic for *Aspergillus niger* in the highest concentration employed

(198×10^{-5} M). The minimal fungistatic concentration for *Aspergillus niger* was 24.75×10^{-5} M. Excesses of cysteine, thioglycolate, or bisulfite failed to change the end points of this quinone for either of these molds. As in antibacterial tests, an excess of *l*(-)-cystine had little or no suppressing activity on antifungal properties of either 2-methyl-1,4-naphthoquinone or 2-methyl-3-methoxy-1,4-naphthoquinone.

Nitroprusside tests for sulfhydryl groups were somewhat difficult to read because of the formation of highly colored precipitates in mixtures of sulfhydryl compounds with 2-methyl-1,4-naphthoquinone or 2-methyl-3-chloro-1,4-naphthoquinone. Weakly positive tests might have been masked. As nearly as could

TABLE 5

Nitroprusside tests for sulfhydryl groups in mixtures of quinones and l(+)-cysteine HCl

QUINONE	CONC. (M)	TEST FOR—SH GROUP	
		Cysteine— 3.33×10^{-3} M	Cysteine— 1.66×10^{-3} M
2-Methyl-1,4-naphthoquinone	4.64×10^{-3}	0 (heavy purple ppt)	0 (heavy purple ppt)
	2.32×10^{-3}	+ (purple ppt)	0 (purple ppt)
	1.16×10^{-3}	+ (purple ppt)	+ (weak purple ppt)
2-Methyl-3-chloro-1,4-naphthoquinone	3.87×10^{-3}	0 (lavender ppt)	0 (lavender ppt)
	1.93×10^{-3}	+ (weak lavender ppt)	0 (lavender ppt)
	$.968 \times 10^{-3}$	+	+ (weak)
	$.48 \times 10^{-3}$		+ (weak)
2-Methyl-3-methoxy-1,4-naphthoquinone	3.96×10^{-3}	+ (no ppt)	+ (no ppt)
	1.98×10^{-3}	+ (no ppt)	+ (no ppt)
	$.99 \times 10^{-3}$	+ (no ppt)	+ (no ppt)
0		+	+

be determined in the supernatant fluids of such mixtures, however, an excess of either of these quinones prevented the color due to —SH groups, but an excess of the methoxy quinone neither formed a precipitate with sulfhydryl compounds nor interfered with the positive sulfhydryl test, even after 20 hours' incubation of the mixture at 37 C.

DISCUSSION

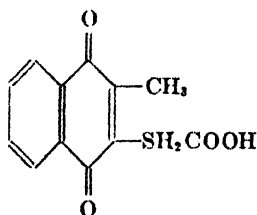
It is evident that 2-methyl-1,4-naphthoquinone inhibits the growth of both bacteria and fungi. After a 4- to 5-day period of incubation for bacterial tests and a 14-day period for molds, minimal inhibitory concentrations for any one test organism were usually the same as minimal killing concentrations with this quinone and also with the chloro quinone. The effect of the methoxy quinone, however, was largely inhibitory; little killing occurred, even with comparatively high concentrations, except with mold 517.

Antagonism of sulfhydryl compounds for antibacterials has been noted by other investigators. Fildes (1940) found that they neutralize the antibacterial

properties of mercury antiseptics, and Nungester, Hood, and Warren (1943) suggested a practical application of this finding in testing disinfectants of this nature. Various groups of recent investigators have reported sulfhydryl compounds to be antagonistic to the action of a variety of antibiotic substances including penicidin (Atkinson and Stanley, 1943); penicillin (Cavallito and Bailey, 1944; Hickey, 1945; Muir and Valley, 1945; Lawrence, 1945); citrinin, gliotoxin, pyocyanin, and several from plants (Cavallito and Bailey, 1944); and clavacin and penicillic acid (Cavallito and Bailey, 1944; Geiger and Conn, 1945). Cavallito and Bailey (1944) believe that many antibiotic substances function chiefly because of their ability to interfere with the sulfhydryl groups concerned in bacterial metabolism. Geiger and Conn (1945) express much the same thought about clavacin and penicillic acid and some synthetic α,β -unsaturated ketones which are also antibacterial. They point out that the $-\text{CH}=\text{C}-\text{C}=\text{O}$ group is the only structural

detail common to these substances, all of which are inactivated by sulfhydryl compounds, and suggest that their antibacterial properties are due to their reaction with $-\text{SH}$ groups in essential bacterial enzyme systems or with $-\text{SH}$ -containing essential metabolites.

2-Methyl-1,4-naphthoquinone may also be considered an α,β -unsaturated ketone. The addition of $-\text{SH}$ compounds takes place with ease at the 3-position on the quinone ring, forming in the case of thioglycolic acid the following quinone acid (Fieser and Fieser, 1944):



S-(2-methyl-1,4-naphthoquinonyl-3)-thioglycolic acid

The position on the quinone ring taken by sodium bisulfite in the formation of the addition product of 2-methyl-1,4-naphthoquinone is not known with certainty. A naphthoquinone having a stable group in the 3-position, such as 2,3-dimethyl-1,4-naphthoquinone, will not form an addition product with bisulfite, indicating that the 3-position is involved in the formation of the addition product. The antagonism of excess bisulfite for the antibacterial activity of 2-methyl-1,4-naphthoquinone suggests that the addition of bisulfite to quinone may follow the same pattern as that involved in the addition of $-\text{SH}$ compounds. Regeneration of quinone from the bisulfite compound in alkali renders the nitroprusside test valueless for determining, in a mixture of addition product and cysteine, whether or not the 3-position of the addition product ring is free to combine with $-\text{SH}$ groups.

The significance of the reducing properties of sulfhydryl groups in suppressing antigrowth properties of 2-methyl-1,4-naphthoquinone has not been conclusively demonstrated in these experiments. The reducing potentials of sodium thiosul-

fate and potassium formate are not equivalent to that of the sulfhydryl group; although positive results with these two compounds might have tended to confirm the importance of reduction, negative results are inconclusive. It may well be that a sharp distinction cannot be drawn between reducing properties and other properties of sulfhydryl groups.

The well-known importance of —SH groups in cell metabolism and the antagonism of sulfhydryl compounds for the antibacterial and antifungal activities of 2-methyl-1,4-naphthoquinone suggest that the antigrowth properties of this quinone function in a manner similar to that suggested for many antibiotic substances, that is, by combining with essential —SH bacterial enzymes or metabolites. It would appear from the results herein reported that the reaction between —SH compounds and 2-methyl-1,4-naphthoquinone blocks a site important in antibacterial and antifungal potency. The methoxy group in the 3-position apparently offers a hindrance to the addition of —SH compounds at this site. The methoxy quinone, however, is definitely inhibitory to the growth of bacteria and molds and, in sufficiently high concentration, will kill at least mold 517 in the presence of excess sulfhydryl. This indicates either that antibacterial and antifungal properties of the methoxy quinone function by a mechanism different from that proposed for the same properties of 2-methyl-1,4-naphthoquinone, in spite of the structural similarity of the two compounds, or that the proposed mode of action of the latter is only one of the means by which it exerts its effects.⁴

SUMMARY AND CONCLUSIONS

Excesses of —SH compounds such as sodium thioglycolate, cysteine, and ethyl mercaptan and of reducing agents such as sodium bisulfite and sodium hydrosulfite suppress antibacterial and antifungal properties of 2-methyl-1,4-naphthoquinone and antibacterial properties of 2-methyl-3-chloro-1,4-naphthoquinone. The weaker reducing agents, sodium thiosulfate and potassium formate, have no effect on antibacterial properties.

Excesses of —SH compounds and bisulfite have little or no effect on minimal effective antibacterial and antifungal properties of 2-methyl-3-methoxy-1,4-naphthoquinone.

Mixtures of cysteine or thioglycolate with excesses of 2-methyl-1,4-naphthoquinone or 2-methyl-3-chloro-1,4-naphthoquinone form colored precipitates, and —SH groups cannot be detected by nitroprusside tests on such mixtures. An excess of 2-methyl-3-methoxy-1,4-naphthoquinone forms no precipitate with cysteine or thioglycolate, however, and positive nitroprusside tests are obtained.

These findings suggest that the mechanism of antibacterial and antifungal action of 2-methyl-1,4-naphthoquinone is by blocking —SH enzymes or metabolites essential for growth. The inhibitory potency of the methoxy quinone, however, even in the presence of sulfhydryl compounds, and its failure to interfere with —SH tests indicate either that the methoxy quinone, in spite of its struc-

⁴ Brief tests with 2,3-dimethyl-1,4-naphthoquinone have indicated that its antibacterial activity is largely inhibitory in nature rather than bactericidal. In this compound the 3-position is also blocked by a stable group, possibly suggesting that bactericidal and bacteriostatic activities are dependent on different mechanisms.

tural similarity, functions by a different mechanism, or that the foregoing explanation for the activity of 2-methyl-1,4-naphthoquinone is incomplete.

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THE INHIBITORY ACTION OF SALIVA ON THE DIPHTHERIA BACILLUS: THE ANTIBIOTIC EFFECT OF SALIVARY STREPTOCOCCI

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The resistance to infection of the tissues of the mouth greatly impressed early workers, who suspected an antibacterial action of saliva and devised experiments to investigate this possibility. (For complete references see Thompson, 1940.) Up to 1934 the results obtained were indecisive, and there was disagreement as to the presence or importance of such antibacterial action. In 1934 and succeeding years Dold and Weigmann (1934), Dold (1935), Dold, Lachele, and Hsing (1936), Weigmann and Koehn (1936), and Weigmann and Noeske (1937) introduced plating methods and showed quite conclusively that saliva had an inhibitory action against the diphtheria bacillus. The Rochester school, Clough (1934), Taylor and Bibby (1935), Hine (1936), Bibby and Ball (1937), Bibby (1938), Clough, Bibby, and Berry (1938), Bibby, Hine, and Clough (1938), and Kesteren, Bibby, and Berry (1942), demonstrated a definite action against lactobacilli and other organisms. In this paper we are concerned only with the inhibitory action of saliva against the diphtheria bacillus. Dold and his co-workers arrived at no definite knowledge as to the nature of the agent active against the diphtheria bacillus but did present considerable evidence that the salivary bacteria were not concerned. In a previous report Thompson (1941) showed that the agent could not be lysozyme since it could be destroyed or removed from the saliva under conditions in which all the lysozyme was retained or even concentrated.

In view of the findings reported in this paper it seemed desirable to review in some detail the evidence presented by the earlier workers as to the role of salivary bacteria in the inhibitory action of saliva. The results of attempts to destroy or remove the bacteria of saliva while retaining the inhibitory action have been conflicting. Dold, Lachele, and Hsing (1936) and Weigmann and Noeske (1937) found that the inhibitory agent was removed by filtration, but Weigmann and Koehn (1936) and Casassa (1937) reported that the agent passed both Seitz and Berkefeld filters. Weigmann and Noeske (1937) reported that prolonged centrifugation removed the agent but assumed that the loss was due to the temperature (64 C) reached in the centrifuge. The same authors found that metallic copper kept in the saliva for long periods at 37 C killed off most of the bacteria but left the antibacterial power unaffected. The fact that this power could later be destroyed by heat indicated to them that they were not dealing with the oligodynamic effect of copper ions transferred in the saliva. Experiments attempting to show whether or not salivary bacteria have an inhibitory effect on

the diphtheria bacillus have also given contradictory results. Weigmann and Noeske (1937) found that some salivary streptococci and staphylococci had a slight inhibitory action on the diphtheria bacillus but stated that many more organisms than were present in saliva were necessary to produce this action. Besta and Kuhn (1934-35) reported that viridans and hemolytic streptococci from saliva inhibited the diphtheria bacillus. Weigmann and Holzl (1940) and Holzl (1941) more recently studied the antagonistic action of mouth bacteria against the diphtheria bacillus. They found strains of hemolytic and viridans streptococci to be inhibitory but concluded that these organisms could not be mainly responsible for the antibacterial power of saliva since very large numbers of organisms were necessary to produce the action; the property was quickly lost in culture and could be abolished by the presence of other bacteria. Indirect evidence against the role of bacteria was given by Dold, Lachele, and Hsing (1936) and by Weigmann and Noeske (1937), neither group finding any correlation between the potency of the inhibitory action of saliva and the numbers of bacteria present.

METHODS

Except where otherwise noted, all tests for inhibition were made by placing standard drops of the material to be tested on the surfaces of agar pour plates containing various concentrations of diphtheria bacilli and by observing the zones of inhibited growth around these drops after a suitable incubation period. The *Corynebacterium diphtheriae* used as the test organism is a strain isolated several years ago from a typical case in this hospital and maintained since then by frequent transfers on rabbits' blood agar. It has the cultural characteristics of an intermedius type and produces a toxin with characteristic action on guinea pigs. The toxin is neutralized specifically by commercial diphtheria antitoxin. Sixteen- to nineteen-hour cultures of the organism on rabbits' blood tryptose agar slants were emulsified in 3 ml of 2 per cent tryptose (Difco) solution, and several serial dilutions were made in the same medium. As noted below, certain optimal concentrations of the organisms were necessary for the demonstration of maximal inhibition. Since optimal dilutions varied from time to time, two different concentrations were used in all experiments. The desired dilutions of the organisms were added to the agar medium at 45 C and thoroughly mixed; plates containing exactly 13 ml of the agar were poured. The agar medium used had the following composition: 1.5 per cent agar, 0.3 per cent meat extract (Difco), 0.5 per cent NaCl, and 0.3 to 0.5 per cent tryptose (Difco), adjusted to pH 7.2. As noted below, the concentration of tryptose was important and the optimal concentration varied somewhat with each new bottle. After the plates hardened they were inverted and placed in the refrigerator for 1 hour.

The saliva or other material to be tested was dropped onto the agar surface from a 27-gauge needle on a syringe held vertically about 2 inches above the plate. It was found that 5 drops could be satisfactorily tested on one plate. All tests were done in duplicate and frequently in triplicate. The drops were allowed to dry at room temperature with the plate covers slightly displaced.

The plates were then inverted and placed in a 37 C incubator. After incubation for 18 to 48 hours they were carefully examined in an indirect, strong light with a dark background. The widths of the zones around the drops, in which no growth of the diphtheria bacillus occurred, were carefully measured and recorded (figures 1 and 2).

Other details of the method depended on the nature of the particular experiment and will be described in the appropriate sections.

PRELIMINARY OBSERVATIONS ON FACTORS AFFECTING THE INHIBITORY
ACTION OF SALIVA ON *C. DIPHTHERIAE*

The work on the inhibitory action of saliva reported previously was done in New York using infusion agar. On returning to the problem here, some months later, it was very difficult, at first, to show any action at all with most salivas. In view of the possibility that the 2 per cent tryptose agar which we were now using might be too rich a medium, experiments were done in which various con-

TABLE 1

Effect of variations in the numbers of diphtheria bacilli on the inhibitory action of saliva.

DILUTIONS OF BACILLARY SUS- PENSIONS IN PLATES		SALIVA DILUTIONS											
		Undiluted			1:10			1:100			1:1,000		
1:1,000	Widths in mm of zones of	0	0	0	3	3	2	5	2	2	2	1	1
1:10,000	inhibition around drops	0	0	0	12	12	15	12	10	10	10	10	10
1:100,000	of saliva dilutions	0	0	0	20	15		20	15		15	8	

centrations of tryptose were used, the other ingredients remaining the same. No inhibition could be detected on the plates containing the usual amount (2 per cent) of tryptose. The largest zones and inhibition in the greatest saliva dilutions were found in the plates containing 0.2 per cent tryptose. In lower concentrations the growth of the diphtheria bacilli was too irregular. Numerous experiments of this type gave similar results (table 7), with the exception that the optimal concentrations of tryptose varied somewhat with each lot of this material. It was more often 0.3 or 0.4 per cent than 0.2 per cent as in this particular experiment. Variations in the concentration of beef extract in the medium had a very much smaller effect on the inhibition than had the variations in tryptose.

Variation in the numbers of *C. diphtheriae* inoculated into the agar medium produced considerable differences in the inhibitory power of the saliva drops. With fewer organisms wider zones of inhibition were obtained and inhibitory action was demonstrated with more dilute saliva. A typical experiment is shown in table 1.

With a hundredfold dilution of the organisms in the plates the widths of the inhibition zones were increased three and four times. With excessive dilutions of the suspensions of bacilli the demarcation between growth area and inhibition

area became indistinct and accurate measurements impossible. In table 1 it is noted that the undiluted saliva had no inhibitory action, but the same saliva when diluted was active (figure 1). This phenomenon has been noted frequently with many different salivas and, as will be discussed in detail below, is due to the presence in the saliva of certain organisms which antagonize the inhibitory action.

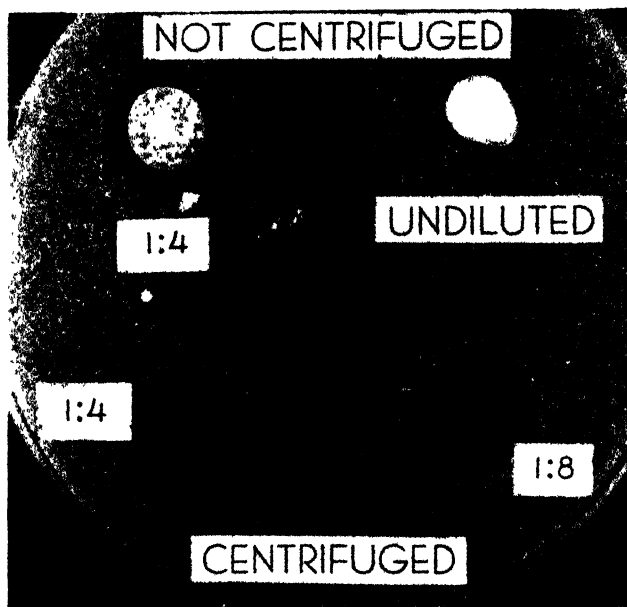


FIG. 1. EFFECT OF DILUTION AND CENTRIFUGATION ON THE INHIBITORY POWER OF SALIVA

Upper left - uncentrifuged saliva diluted 1:4
Upper right - uncentrifuged undiluted saliva.
Lower right - centrifuged saliva diluted 1:8.
Lower left - centrifuged saliva diluted 1:4

THE INHIBITION OF *C. DIPHTHERIAE* BY SALIVARY STREPTOCOCCI

During a long series of unsuccessful attempts to concentrate and purify the active agent in saliva, the possibility of a bacterial factor was ignored. We were not at that time aware of the reports of Weigmann and Holz (1940) and Holz (1941), and probably attached too much weight to the negative evidence of the previous workers. It was finally forced on our attention that occasionally very definite zones of inhibition occurred around certain colonies growing within the area of the saliva drops. Pure cultures in broth of these organisms produced marked inhibition when dropped on the plates in the same manner as the saliva dilutions (figure 2).

One hundred and fifteen strains of organisms were isolated in pure cultures at different times from the actively inhibitory salivas of 12 individuals. Eighteen- to twenty-four-hour cultures in 2 per cent tryptose broth were tested for inhibitory action on the diphtheria bacillus. The cultures were also studied on rab-

bits' blood tryptose agar plates, and gram-stained preparations were examined. The results of these studies are summarized in table 2.

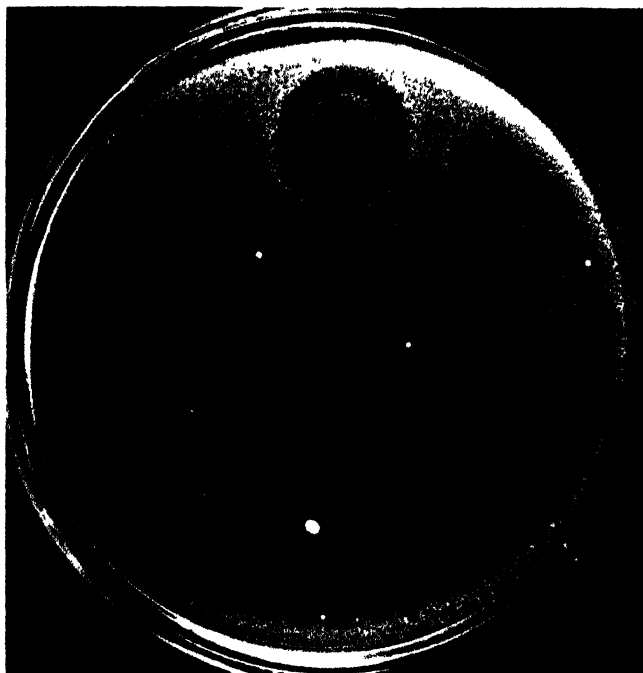


FIG. 2. INHIBITION OF DIPHThERIA BACILLI BY DILUTIONS OF PURE CULTURE OF MITIS TYPE VIRIDANS STREPTOCOCCUS

The opaque colony showing inhibition is an aerial contaminant

TABLE 2

Inhibition of C. diphtheriae by salivary organisms

TYPE OF ORGANISM	NUMBER OF STRAINS TESTED	NUMBER OF STRAINS PRODUCING DEFINITE INHIBITION
Streptococci showing definite green zones on blood agar	55	48*
Indifferent streptococci	28	3†
Streptococci showing beta hemolysis on blood agar	7	1
Coagulase negative staphylococci and sarcinae	16	0
Coagulase positive staphylococci	1	0
Diphtheroids	8	0

* Some negative strains became positive on later transfer.

† One strain showed green zones on later transfer.

These tests were on organisms isolated, more or less at random, from the growth occurring on saliva drops which showed active inhibition, and the distribution or numbers of organisms have of course no necessary relation to the distribution or numbers of organisms in saliva.

Since all the active organisms were streptococci, further studies were made on these streptococci using the methods suggested by Sherman (1937) and Sherman, Niven, and Smiley (1943). Twenty-seven inhibitory strains and twenty-two noninhibitory strains were tested as to the following properties: growth in 6.5 per cent sodium chloride; growth on 30 per cent bile; growth at 45 C; the production of mucoid colonies on 5 per cent sucrose agar; and the amount of acidity produced in glucose broth. The results of these tests are given in table 3. The organisms are grouped according to the types indicated by the results of the tests.

The great majority of actively inhibitory strains were of Sherman's mitis type; only 4 of the 22 inactive strains were of this type. Only one of the sali-

TABLE 3
Properties of inhibitory and noninhibitory streptococci

	INHIBITORY STRAINS	NONINHIBITORY STRAINS
Number of strains studied	27	22
Number of strains with "mitis" properties: No growth in 30% bile No growth in 6.5% salt No mucoid colony on 5% sucrose pH in glucose above 4.4 Definite green zones on blood agar	24	4
Number of strains with "salivarius" properties: No growth in 6.5% salt Mucoid colonies on 5% sucrose pH in glucose 4.4 or below	1 (showed definite green zones on blood agar)	16
Number of strains with "enterococcus"* properties: Growth in 6.5% salt Growth on 30% bile	2 (1 showed definite green zones on blood agar)	2 (1 showed beta hemolysis)

* Many enterococci isolated from feces inhibited the diphtheria bacilli, but we are concerned here with organisms isolated from saliva. Several pneumococcal strains of different types studied have shown marked inhibition.

varius type strains inhibited the diphtheria bacillus, and it was atypical in producing marked green zones on blood agar.

ATTEMPTS TO REMOVE THE STREPTOCOCCI WHILE PRESERVING THE INHIBITORY ACTION OF SALIVA

Filtration. Thompson (1941) showed that the antidiphtheria agent in saliva did not pass filters when the saliva was acidified, although the lysozyme of saliva was filterable under these conditions. To determine whether the inhibitory agent would pass filters without acidification of the saliva, 25- to 50-ml volumes of saliva were diluted with equal parts of 2 per cent tryptose solution, centrifuged briefly to remove coarse particles, and filtered through Seitz, Mandler, and Berke-

feld W and N filters. Various fractions of the filtrates were tested for antibacterial activity by the drop-on-plate method described above. The unfiltered, diluted salivas served as controls. In no case was any inhibition of the diphtheria bacillus produced by the filtered salivas in spite of the high activity of the unfiltered materials.

It was possible that the plate method was not sensitive enough to detect small amounts of the agent which might pass the filters. Since the sterility of the filtrates made a tube method of testing for inhibition possible, several experiments were done in which filtrates were inoculated with various numbers of diphtheria bacilli and the presence or absence of growth was observed. A typical experiment follows: 25 ml of fresh saliva were diluted with equal parts of 2 per cent tryptose broth and centrifuged at 1,800 rpm for $\frac{1}{2}$ hour. Two ml of the supernatant were preserved for control tests, and the remainder was filtered through a Seitz disk at 19 pounds' pressure. The first and second portions of the filtrate were kept separate. The filtrates were distributed in small tubes in 1.5-ml

TABLE 4
No inhibition of diphtheria bacilli by Seitz filtrates of saliva

MEDIUM IN TUBES	RELATIVE GROWTHS OF BACILLI IN VARIOUS TUBES						WIDTH IN MM OF INHIBITION ZONES ON PLATES					
	3+	3+	3+	3+	3+	2+						
Saline + 2% tryptose	3+	3+	3+	3+	3+	2+						
Saliva filtrate 1st half + 2% tryptose	4+	4+	4+	4+	4+	4+	0	0	0	0	0	0
Saliva filtrate 2nd half + 2% tryptose	4+	4+	4+	4+	4+	4+	0	0	0	0	0	0
Saliva unfiltered							2	5	3	2	5	6
Dilution of diphtheria bacilli inoculated	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸					10 ⁶

amounts. Similar tubes of the broth diluted with equal parts of saline were prepared. The tubes were then inoculated with standard drops (from a 27-gauge needle) of various dilutions of a suspension of an 18-hour culture of the diphtheria bacillus. They were incubated for 18 hours, and the relative amounts of growth were determined by inspection and by microscopic examination of stained preparations. The filtrates and the unfiltered saliva dilutions were also tested by the standard "drop plate" method. The results are shown in table 4.

Similar results were obtained in a duplicate experiment with saliva from a different person and in other experiments using 0.1 per cent tryptose as the diluting medium to eliminate the possibility of the 2 per cent tryptose antagonizing the inhibitory action. Filtered saliva-saline mixtures with no added tryptose also supplied adequate nutrition for the diphtheria bacilli and supplied good growth, indicating clearly that any inhibitory agent had been removed by filtration. Filtration through Mandler filters with tryptose or saline diluents gave similar results. Up to 90-ml volumes of diluted saliva were filtered with no evidence of any inhibitory activity in the filtrate. The saliva filtrate invariably tended to produce better growth than similar broth controls.

Bactericidal effect of copper. The report of Weigmann and Noeske (1937) that they succeeded in killing most of the bacteria of saliva without affecting the antibacterial power has already been referred to. Several unsuccessful attempts were made to repeat this work. Fresh saliva was diluted with equal parts of saline and centrifuged at 1,800 rpm for $\frac{1}{2}$ hour. The supernatant was placed in a tube so as just to cover a coiled 20-gauge copper wire. The coils were such as to get 26 inches of wire into $1\frac{1}{4}$ inches of a tube $\frac{1}{2}$ inch in diameter. A small portion of the centrifuged diluted saliva was kept in the refrigerator to be used to control the original inhibitory activity. The saliva containing the copper was incubated at 37 C, and samples were removed after 24 and 72 hours. They were compared with the refrigerated material as to inhibitory potency by the usual drop plate method. The samples had no antibacterial activity demonstrable by this method although the refrigerated material was very active. Cultures of the treated saliva showed no growth of any organisms.

TABLE 5
Effect of centrifugation on inhibitory activity of saliva supernatants

SPEED AND TIME OF CENTRIFUGATION OF SALIVA	WIDTHS OF ZONES OF INHIBITION IN MILLIMETERS	
Uncentrifuged control	5	5
700 rpm 5 minutes	6	6
700 rpm 15 minutes (in addition to above)	8	8
1,400 rpm 30 minutes (in addition to above)	4	4
1,400 rpm 45 minutes (in addition to above)	2	2
1,700 rpm 90 minutes (in addition to above)	0	0
1,700 rpm 120 minutes (in addition to above)	0	0

Centrifugation experiments. Many experiments using centrifugation for different time periods at various speeds were done with salivas from several different individuals. With the exception of certain apparently anomalous reactions, described and explained below, the results of all the centrifugation experiments were entirely in accord with the concept that the inhibitory action of saliva is due to certain of the bacteria contained in it. With the exception noted, the more rapid and more prolonged the centrifugation the more inhibitory activity was lost from the supernatant fluids. In several instances 2,000 rpm for $1\frac{1}{2}$ hours completely removed all activity from the supernatants, but the sediments were very active in considerable dilutions. Since the sediments were still active, the temperature reached in the centrifuge could not have been responsible for the loss.

In table 5 are shown the results of an experiment in which only the supernatants were tested. Ten ml of saliva were diluted with equal parts of saline and centrifuged at the speeds and for the periods indicated. Samples of supernatant were removed at the various times and tested by the drop plate method without further dilution.

An increased inhibitory action of the saliva after brief centrifugation was evi-

dent. The phenomenon was demonstrable in practically all cases when saliva, undiluted or moderately diluted, was centrifuged at relatively slow speeds for short periods (800 to 1,000 rpm for 5 to 20 minutes). (See figure 1.) The differences, in many experiments, were more marked than in the one charted. In one experiment, for example, the centrifuged saliva produced zones of inhibition 8, 9, and 10 mm in width, whereas the corresponding dilution of the uncentrifuged material showed 2-, 3-, and 2-mm zones. This phenomenon of increased action of the supernatant after brief centrifugation is comparable to the increase with moderate dilution noted previously, and, as will be shown later, is likewise due to the elimination of certain other bacteria which antagonize the inhibitory power of the streptococci. The sediments from the centrifuged salivas showed increased inhibitory activity on moderate dilution in the same manner as the untreated salivas, but the supernatants which retained any activity lost it progressively on dilution.

Effect of heat. It was previously shown (Thompson, 1941) that heating saliva to 100 C° at an acid pH destroyed the inhibitory action against the diphtheria bacillus but did not affect the lysozyme. To determine whether the inhibitory action of saliva could be preserved while the streptococci were destroyed, or vice versa, a number of experiments were done to determine the effect of 56 C° for various periods. Dilutions of saliva and pure cultures of active streptococci were heated in a water bath at 56 C°; samples were removed at various periods, serially diluted, and tested by the standard technique on plates containing diphtheria bacilli. In both cases all inhibitory action was destroyed in from 5 to 7 minutes. In both cases also the active streptococci were destroyed in approximately the same periods, slight differences being explained by the survival of a small number of organisms insufficient to produce visible inhibition.

ANTAGONISM OF INHIBITORY ACTION OF SALIVA BY OTHER MOUTH BACTERIA

The increase of inhibitory action on moderate dilution or centrifugation of saliva was always associated with a diminution in the number of raised, opaque bacterial colonies growing from the drops on the test plates (figure 1). The active streptococci formed small, grayish, flat, translucent colonies on the medium used. This suggested that the organisms forming the opaque colonies in some way antagonized the inhibitory action of the green streptococci. Being greatly in the minority as compared to the streptococci, the antagonistic organisms were reduced by dilution or centrifugation below a critical concentration before the inhibitory streptococci were so reduced. When isolated in pure culture the opaque colonies were shown to be staphylococci, in most cases white coagulase-negative strains but occasionally orange and coagulase-positive ones. It was shown that pure cultures of these staphylococci did antagonize the inhibitory action of saliva. Tests of saliva dilutions on the drop plates were prepared in the usual way, and the pure cultures of staphylococci were inoculated directly onto the drops on the plates and the plates dried and incubated. The results of one such experiment are shown in table 6.

Additional similar experiments gave similar results. Many strains of staphylococci and sarcinae produced such antagonism of the inhibitory activity of saliva, but diphtheroids¹ and salivarius type streptococci did not antagonize the inhibition. Both pathogenic and nonpathogenic staphylococci were effective antagonists. Pure cultures of inhibitory streptococci were antagonized in the same manner by the same organisms as was saliva. The mechanism of the

TABLE 6

Antagonism by pure cultures of staphylococci of salivary inhibition of diphtheria bacilli

TYPE OF STAPHYLOCOCCUS CULTURE INOCULATED ONTO DROPS	DILUTIONS OF SALIVA USED IN TEST DROP	WIDTH OF ZONES OF INHIBITION
None	1:8	15 mm
None	1:16	13 mm
None	1:32	12 mm
White, coagulase-negative	1:8	4 mm
White, coagulase-negative	1:16	0
White, coagulase-negative	1:32	0
Orange, coagulase-positive	1:8	0
Orange, coagulase-positive	1:16	0
Orange, coagulase positive	1:32	0

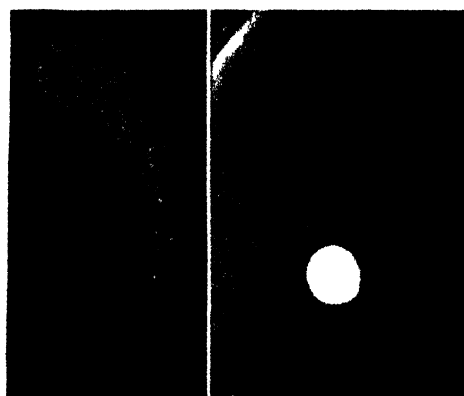


FIG. 3. NO. 1. (LEFT) EFFECT OF ALLOWING DROP OF UNDILUTED SALIVA TO RUN DOWN TEST PLATE
NO. 2. (RIGHT) ANTAGONISM OF INHIBITORY EFFECT OF PURE CULTURE OF STREPTOCOCCUS BY STAPHYLOCOCCUS (OPAQUE WHITE GROWTH)

antagonism has not been adequately studied but the available evidence indicates that it is not the result of an inhibition of the growth of the active streptococci. When the antagonizing organisms were inoculated close to a drop of the inhibitory streptococci, the zone of inhibition of the diphtheria bacilli was abolished in the area but the growth of the streptococci was not apparently

¹ Several diphtheroid strains isolated from saliva were themselves susceptible to inhibition by the streptococci.

affected (figure 3, no. 2). No evidence of inhibition of the active streptococci by the antagonistic organisms was obtained using the drop plate technique with the streptococci in the plates and the antagonizing organisms in the drops. It would appear that the antagonizing organisms either prevented the formation of the inhibitory agent by the streptococci, or neutralized it after it was formed. The relationship between the inhibiting and antagonizing organisms was quantitative: if too many inhibitory streptococci were present no antagonism could be demonstrated. In some instances when the concentration of tryptose in the medium was just below that necessary for good growth of the diphtheria bacilli, it was noted that these organisms grew well around colonies of staphylococci which were antagonistic to inhibition. It is possible but not proved that the staphylococci antagonized the inhibition by supplying some growth factor the absence of which made inhibition by the streptococci possible.

Other phenomena apparently very peculiar when first observed were explained by the antagonistic action of the salivary staphylococci. In many instances when drop plate tests of saliva were done and the plates were not kept level, the saliva drops ran across the plate for some distance. Frequently when this occurred, the zones of growth inhibition were very much wider at the end of the run than around the original area of the drop. The opaque staphylococcal colonies in the drop were almost always concentrated on the original area and obviously antagonized the inhibition in that area, whereas the active streptococci were present in the "run down" position in more or less pure culture (figure 3, no. 1). Another observation was made during some early attempts to determine whether the supposed chemical inhibitory agent was of a dual nature. When fresh, active salivas were added to saliva heated to 56 C (5 to 15 minutes), the fresh salivas frequently were inactivated by the heated saliva. The active streptococci in the heated saliva had been killed, but the more resistant staphylococci were still present and antagonized the inhibitory action of the streptococci in the fresh saliva. When the heated saliva was centrifuged at 1,000 rpm for 10 minutes, the supernatant lost both its power to antagonize and its staphylococci, which were then in the sediment.

INHIBITORY PROPERTY OF SALIVA AND OF STREPTOCOCCAL CULTURES AFFECTED BY THE SAME FACTORS

Indirect evidence that the inhibitory property of saliva was due to the streptococci present in it was given by the fact that a number of different conditions affected fresh saliva and pure cultures of active streptococci in the same fashion.

It was shown above that the tryptose content of the medium used in the tests had a marked influence on the inhibition of diphtheria bacilli by saliva. Variation in the tryptose concentration affected the inhibitory action of pure cultures of streptococci in the same manner. Table 7 shows an experiment in which various dilutions of saliva and of a pure culture of a mitis streptococcus were tested at the same time on plates containing various concentrations of tryptose.

It has been indicated above that the inhibitory powers of cultures of streptococci and of saliva are both antagonized by certain other salivary bacteria.

During some early attempts to determine whether the supposed chemical inhibitory agent in saliva was of a dual nature, it was noted that dilution of the active, fresh saliva in saliva heated to 95 C for 15 minutes, rather than in saline, definitely increased its inhibitory action. Dilution in 2 per cent tryptose had a similar effect. Likewise, the inhibitory action of pure cultures of mitis streptococci was greater when dilutions were made in saliva heated to 95 C or in 2 per cent tryptose solution. The increased inhibitory action with both saliva and streptococcal cultures was associated with a greater growth of streptococci in the nutrient diluents, particularly in the more dilute preparations.

TABLE 7

Parallel effect of variation in tryptose content of medium on inhibitory action of saliva and of streptococcal culture

DILUTION OF AGENT		TRYPTOSE IN MEDIUM			
		0.3%	0.5%	1%	2%
Streptococcus					
1-160	Widths of zones of inhibition in millimeters	Diphtheria bacilli too sparse for accurate measurements	8	6	1
1-320			8	5	0
1-640			11	3	0
1-1,280			9	3	0
1-2,560			9	1	0
1-5,120			5	+	0
Saliva					
1-16			12	6½	0
1-32			10	7	0
1-64			5	1	0
1-128			3	0	0
1-256			2½	0	0
1-512			+	0	0

DISCUSSION

The demonstration that pure cultures of salivary streptococci of the mitis type inhibited the growth of diphtheria bacilli, together with the failure of all attempts to separate the inhibitory action from the streptococci and the indirect evidence presented (both streptococci and inhibitory action of saliva being affected by the same factors), would seem to be fairly adequate proof that the ability of saliva to inhibit diphtheria bacilli, demonstrable by the technique used in these experiments, is entirely due to the inhibitory organisms present in it. The failure of previous workers to find any relationship between the numbers of salivary bacteria and the inhibitory power of the saliva can readily be explained by the number of factors involved, including the numbers and relative power of the active organisms and the numbers and relative power of the antagonizing organisms. The few reports of successful filtration of the agent cannot be explained on the knowledge available. We cannot agree with the reports of Weigmann and Holz (1940) that extremely large numbers of streptococci are always

necessary to demonstrate the action. Occasionally, zones of inhibition could be demonstrated around single colonies of the streptococci, and the numbers of streptococci required to produce inhibition by pure cultures was not greater than those present in the active saliva dilutions.

SUMMARY

Pure cultures of the *Streptococcus mitis* type of viridans streptococci isolated from saliva inhibited the growth of diphtheria bacilli in the same manner as fresh saliva. The inhibitory action of saliva could not be demonstrated after the streptococci had been removed from it by filtration, centrifugation, heat, or the bactericidal effect of copper. The inhibitory actions of saliva and of pure cultures of mitis streptococci were affected similarly by several factors: the tryptose content of the medium, the antagonistic action of staphylococci, heat, and the use of nutrient materials as diluents.

The inhibitory action of salivary streptococci was best demonstrated when the tryptose content of the medium was between 0.2 per cent and 0.5 per cent, and was abolished in the presence of the normal tryptose content (2 per cent).

Staphylococci antagonized the inhibitory action of salivary streptococci without affecting the growth of these organisms.

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FURTHER NOTES ON THE CHARACTERISTICS OF PROTEUS AMMONIAE

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The characteristics of certain strains of bacteria agreeing with the description of *Proteus ammoniae* (Magath, 1928) have previously been reported (Fulton and Harrison, 1943). It was shown that the species was indole-negative, failed to ferment maltose and mannose, and gave a delayed fermentation reaction in sucrose unless the concentration in the broth is increased considerably.

We are now able to report the results of a study of 110 strains. They can be divided into two groups, called for convenience "typical" and "atypical." The typical group, comprising 74 strains, all conformed exactly to the description recorded in the previous report with the following modifications: (1) All typical cultures were Voges-Proskauer-positive when grown in Difco or BBL MRVP medium for 2 days at 37 C and tested by the method of Vaughn and Levine (1942). (2) Strains differed in their fermentation of cellobiose and fructose. Neither carbohydrate was fermented by 31 strains, 26 fermented fructose but not cellobiose, 16 fermented both carbohydrates, and 1 fermented cellobiose but not fructose. Strain differences in these fermentation tests were constant and reproducible. One of these strains was nonmotile, but it has been classified as typical inasmuch as it conformed to the description in all other tests and the occasional occurrence of such an O form could well be anticipated.

The atypical group included 36 strains which agreed with the typical group in a majority of features but which differed with respect to urea hydrolysis, gelatin hydrolysis, or Voges-Proskauer reaction. Twenty-two of the strains gave negative tests for the utilization of gelatin. Thirty-one were V.-P.-negative. Both the gelatin and the V.-P. tests were negative in 16 strains; 9 of these were lost but those which were available for retesting more than a year later were consistently negative in both tests. Among these atypical strains there were 8 which were positive in both cellobiose and fructose, 11 in fructose alone, 1 in cellobiose alone, and 16 which fermented neither carbohydrate. This was the same order of relative frequency that was seen among the typical strains.

Only two of the 110 cultures were urea-negative. They were atypical also in the V.-P. and gelatin reactions.

One atypical strain which was gelatin-negative had the unique ability to ferment salicin slowly. Another, which was V.-P.-negative, fermented both adonitol and mannose. The fermentation of adonitol is especially noteworthy as few species of bacteria are known to ferment this carbohydrate, the most familiar being Rettger's bacillus (Fulton and Curtis, 1946).

Fulton and Harrison reported that this species was serologically heterogeneous. Of 103 strains tested for agglutination, 27 were positive with a serum for a single

selected strain of *P. ammoniae*. There was no correlation of biotype with serotype, the agglutinable strains being distributed in proportion between the typical and atypical groups. No agglutination was observed in serums for several strains of Morgan's bacillus, nor in serums for single strains of *P. vulgaris* and *P. asiaticus*. These incomplete studies suggest that the species *P. ammoniae* consists of several serotypes but is distinct from the other species of *Proteus*. It is possible that reports of cross agglutination among strains of the various species of *Proteus* may have been based on experiments in which the serums used were derived from strains which in the present investigation have been grouped together as this single species.

DISCUSSION

It can be seen that this collection is fairly homogeneous in its bacteriological properties. The cardinal characteristics of the genus *Proteus* according to Bergey (1939) are swarming, urea hydrolysis, protein decomposition, failure to ferment lactose, and motility. The cultures studied included none which fermented lactose, only one which was nonmotile, and two which failed to attack urea. Otherwise the entire collection possessed these cardinal characteristics. Protein decomposition was exhibited by gelatin hydrolysis, peptonization of milk, and production of H_2S . Nineteen strains failed to attack gelatin and also were non-proteolytic in milk, although all produced H_2S .

Sucrose fermentation is also ordinarily considered to be characteristic of *Proteus*. Fulton and Harrison (1943) showed that in 5 per cent sucrose broth these cultures consistently fermented this carbohydrate. Rustigian and Stuart (1945) called attention to the small gas volume produced by *Proteus* cultures; this characteristic was observed in the present collection of strains.

In characterizing the genus, Bergey further states that the V.-P. reaction is usually negative. In this respect *P. ammoniae* departs from the generic description.

One of the serious difficulties in working with the genus *Proteus* has been uncertainty over how the species lines should be drawn. The tendency to refer to the organism as "*Bacillus proteus*" is now restricted largely to clinical medical literature. Rustigian and Stuart (1945) proposed that four species be accepted: namely, *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri*. They considered *P. ammoniae* to be a variant of *P. mirabilis*. These authors showed that considerable confusion has arisen concerning the characteristics of this species. The original description of *P. mirabilis* by Hauser (1885) gave insufficient data with which to differentiate the species from others now commonly assigned to the genus. The description of *P. ammoniae* (Magath, 1928) is detailed enough for that species to be recognized, although emendations in regard to indole and acetylmethylcarbinol production and the fermentation of sucrose are necessary. It might be better to accept this recognizable species and to follow Topley and Wilson (1936) in considering *P. vulgaris* and *P. mirabilis* as essentially synonymous terms. It is becoming apparent that *Proteus* species have more importance in relation to various disease processes than they have traditionally been assigned.

It should be helpful in defining their relationship to conditions such as diarrhea, urinary tract infections, and the flora of burns to have a small number of species accepted on the basis of recognizable descriptions backed by authentic type cultures.

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AN IMPROVED SLIDE CULTURE TECHNIQUE FOR THE STUDY AND IDENTIFICATION OF PATHOGENIC FUNGI¹

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The microscopic identification of many pathogenic fungi still depends almost wholly upon morphology, especially upon the size, location, and arrangement of individual spores or groups of spores. The usual procedure is to examine teased wet-mount preparations. These have a number of disadvantages, however. The structures depended upon for the recognition of such fungi as *Blastomyces dermatitidis* or *Sporotrichum schenckii*, for example, are often so distorted or disarranged in these teased mounts that identification is made difficult or impossible, particularly for persons unfamiliar with this technique. Slide cultures, on the other hand, when suitably prepared, make possible the definite recognition of these genera, and also help materially with the classification of other varieties of fungi. Use of the available slide culture techniques (Henrici, 1930; Brown, 1942; Lewis and Hopper, 1943), however, is beset with numerous difficulties. None of these methods is suitable for rapid identification of *pathogenic fungi*.

The present paper describes an improved slide culture technique which is relatively easy to carry out and is useful for practical diagnostic work in mycology, as well as for classroom teaching. Since pathogenic fungi grow slowly, and hence may easily be crowded out by more rapidly developing contaminants, provision has been made to exclude such contaminating organisms. The method also makes possible the direct inoculation of the culture slide with a large inoculum. This has been found to be necessary to insure growth of pathogenic species. Special attention also has been given to the nature of the medium for the microculture. Since identification depends to a large degree upon the presence of typical spores, it is important that the medium used stimulate early and abundant sporulation. Lastly, this improved slide culture technique is designed to permit the easy preparation from the culture of stained slides which preserve intact the morphological features of the fungus. These slides are valuable for making photomicrographs and are especially useful for teaching purposes.

MICROCULTURE TECHNIQUE

Culture media. After some experimentation it has been found that best results in forcing early sporulation of pathogenic fungi are obtained with the specially prepared potato glucose agar described below.

Cut 250 grams of whole potato into pieces about the size of walnuts, place in 500 ml of distilled water, and steam for $\frac{1}{2}$ hour. Pour through a wet towel, make

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up to volume, and add to the extract 1.0 per cent glucose and 1.5 per cent agar. No pH adjustment is necessary. Heat until dissolved, tube, and autoclave at 15 pounds for 20 minutes.

Special equipment. The necessary materials for making the microcultures are illustrated in figure 1. The only special piece of equipment necessary is a syringe filled with a paraffin-vaseline mixture for use in making the cover glass supports.

Paraffin and vaseline are melted together in a ratio of 30 to 70 by weight, and the mixture is sterilized in the dry-heat oven. (Such a ratio may have to be varied because of the different grades of paraffin or vaseline available.) This mixture is placed in a sterile 10-ml syringe which has had the tip broken off close

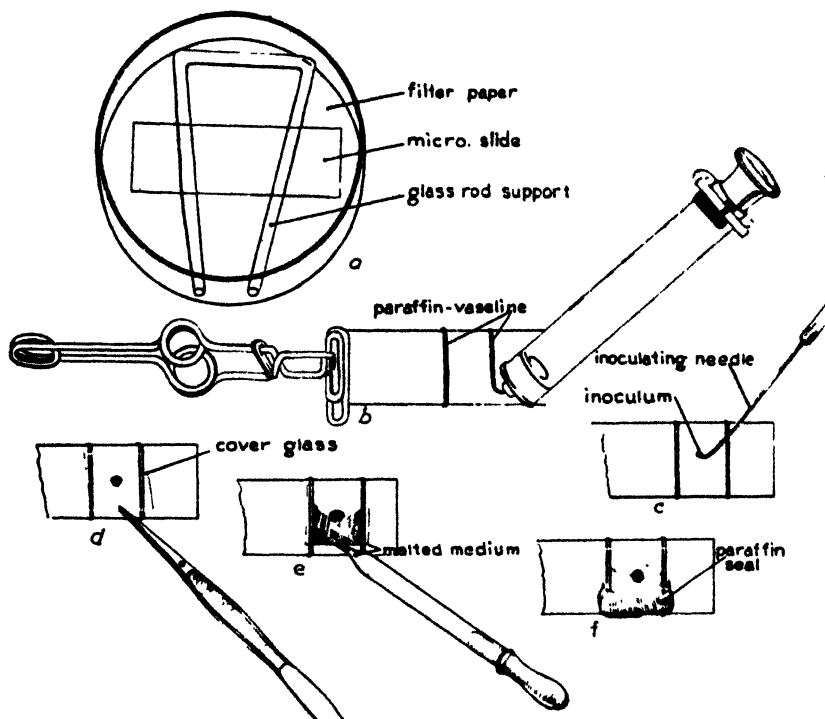


FIG. 1. MICROCULTURE TECHNIQUE

to the barrel in order to obtain a larger opening and to permit easier extrusion of the contents.

Preparation of the microculture. The microscope slide is removed from the sterile petri dish setup, figure 1a, with flamed tweezers, and one end is engaged with the slide forceps.

Two parallel strips of the paraffin-vaseline mixture are extruded out across the slide with the modified syringe, figure 1b, to furnish supports for the sterile cover glass. The slide can now be replaced under the petri dish lid and kept there during the rest of its preparation.

A small piece of fungous growth is "raked" off with a heavy nichrome hooked needle and placed on the center of the slide between the paraffin-vaseline lines,

figure 1c. A sterile cover glass is then pressed on the paraffin-vaseline supports with flamed tweezers to form the desired thickness of space underneath, figure 1d.

By means of a short-tipped, sterile Pasteur pipette, the melted medium (which has been held at 45 C in a constant temperature water bath) is run under the slide up to or just covering the inoculum so that a good "growing edge" is formed, figure 1e. Finally the lower edge is completely sealed with a layer of hot, sterile paraffin applied with a cotton-tipped applicator, figure 1f.

The petri dish setup now acts as a moist chamber for the completed slide culture when sterile water is added to moisten the filter paper. In order to maintain moisture within the dish over long periods of time it has been found helpful to place inside a pledget of sterile, moistened absorbent cotton.

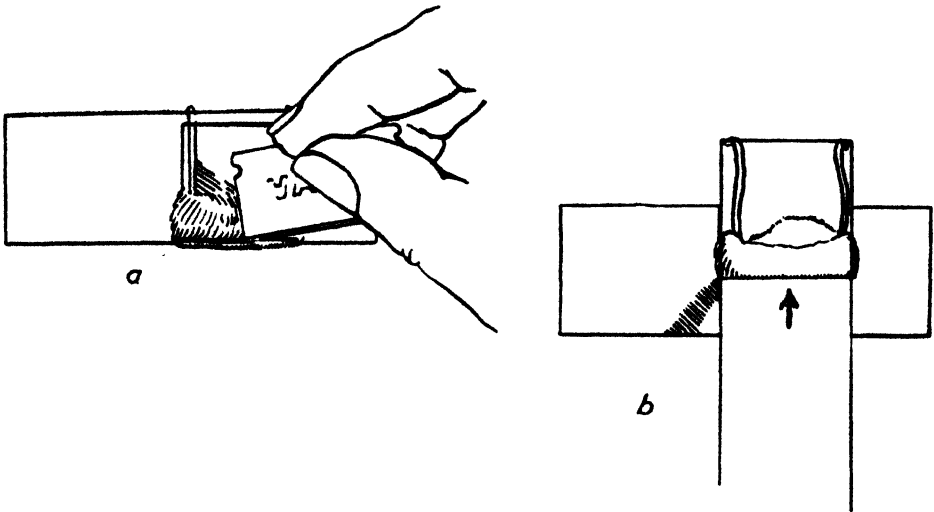


FIG. 2. PREPARATION OF STAINED SLIDES

Preparation of stained slides. The slide cultures are examined every day under the microscope until they have reached the particular phase of growth at which it is desired to make permanent preparations. It will be observed by focusing up and down that there is an adherent growth both on the under side of the cover glass and on the top side of the microscopic slide. Since the growth on the cover glass is usually better than that on the slide, an attempt is made to save the former at the expense of the latter in the procedure which follows.

The culture slide is removed from the moist chamber and the paraffin which seals the agar is cut away with a razor blade, figure 2a. The cover glass with its adherent fungous growth is now slowly pushed forward off the microscopic slide by using the end of another slide, figure 2b. The paraffin-vaseline mixture on the cover glass is then cut away with a razor blade, leaving only the agar and fungous growth. This growth that remains attached to the cover glass is fixed by heating in the flame.

The block of agar still on the cover glass may now be removed simply by lifting it off with a razor blade. However, with fungi that produce a heavy mycelial growth, it is better to cut across the base of the aerial mycelium with a razor blade before lifting off the agar. These manipulations leave the heat-fixed aerial mycelium, with all its sporulating organs, relatively intact on the original cover glass without the interfering presence of the agar. If there is adherent paraffin or vaseline, the cover glass may be dipped into xylol. The preparation is now ready for staining and mounting on a new microscope slide with clarite or balsam in the usual manner.

COMMENTS

This slide culture technique has been used for over two years with consistently good results. It has been employed for all the common genera of filamentous pathogenic fungi and also for several varieties of nonpathogenic molds. Certain points are worth noting, however. It should be emphasized again that a large inoculum is essential in making a slide culture of a pathogenic fungus. It is recommended also that three or four microcultures should be set up at the same time in all cases in order to allow for possible breakage, failure of growth, or failure of the fungus to sporulate fully. The method used to guard against contamination of the slide with unwanted fungi is very effective, as the actual occurrence of such contamination is negligible.

Sabouraud's glucose agar has been used for years by other workers in the preparation of slide cultures, and little effort has been made to secure a medium better suited to the purpose of these cultures, namely, one that will tend to reduce mycelial growth and force early sporulation. Actually, Sabouraud's medium has an effect exactly opposite to that desired. It stimulates such prolific mycelial growth that sporulation is retarded until very late.

It is well known that special media are required to force sporulation of many true yeasts. Some of these media are potato extract agar; carrot juice agar; carrot, cucumber, beet, or potato wedges; Gorodkowa slants; and vegetable extract agar (Mrak, Phaff, and Douglas, 1942). It may be that some of these media will also serve well in microcultures for the purpose of stimulating early sporulations of fungi imperfecti. But preliminary experiments with several of the aforementioned media indicated that a potato glucose agar is more satisfactory than any of them. By using the potato glucose medium described above sporulation occurred with all the common genera of pathogenic fungi in 3 to 14 days as compared to 10 to 41 days with Sabouraud's glucose agar. The character of sporulation was entirely characteristic for each genus, and furthermore the spores were not obscured by heavy mycelial growth. (Commercial dehydrated potato glucose agar was found to be unsatisfactory, since it led to the formation of aberrant spore forms or no spores at all.)

The making of good, stained, permanent mounts from slide cultures requires some practice and ingenuity, but the results are well worth the effort (see figure 3). The stained preparations can be used for teaching students the morphology of the different fungi, and in addition they serve ideally for photomicrographs.



FIG. 3. A, *Microsporium gypseum* ($\times 400$); B, *Trichophyton rubrum* ($\times 900$); C, *Hormodendrum pedrosoi* ($\times 900$); D, *Phialophora verrucosa* ($\times 900$); E, *Coccidioides immitis* ($\times 400$); F, *Blastomyces dermatitidis* ($\times 900$). All cultures grown from 4 to 14 days on potato glucose agar at room temperature. Fixed preparations were stained with 0.5 per cent safranin.

Good results may likewise be obtained with many of the filamentous *non-pathogenic* fungi with this slide culture technique. However, the making of good permanent mounts of many nonpathogenic molds is made difficult by the great tendency of these fungi to release the mature spores with the slightest movement.

A few attempts have been made to use this slide culture method for the primary isolation of pathogenic fungi, as it offered the advantages of easy daily observation of the growth with possible early identification, along with freedom from outside contamination. Two cases of ringworm were studied from which the causative fungi were isolated and identified in this manner—*Microsporum audouinii* in 14 days and *Microsporum canis* in 7 days. It is difficult to say from so limited an experience whether the method is feasible for routine usage in the diagnosis of mycotic infections. It should be pointed out, however, that any medium may be used in these slide cultures, including blood-enriched media.

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SUMMARY

An improved slide culture technique is described for the study and identification of *pathogenic fungi*, in which special provision is made for excluding contaminants. It is relatively easy to prepare.

A new specially devised potato glucose agar is recommended for use with this slide culture for stimulating early and abundant sporulation, in order that the recognition of particular varieties of fungi may be facilitated for the technician and for the student.

A technique is described for making permanent stained mounts from these slide cultures that preserve intact the morphological features of the fungus. These slides are especially useful for teaching purposes and are valuable for making photomicrographs.

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MICROBIOLOGICAL ASPECTS OF PENICILLIN

IX. COTTONSEED MEAL AS A SUBSTITUTE FOR CORN STEEP LIQUOR IN PENICILLIN PRODUCTION

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Since the announcement by Moyer and associates (Coghill, 1944) at the Northern Regional Research Laboratory of the remarkable stimulating effect of corn steep liquor on the production of penicillin by various fungi, this material has been employed almost universally for the production of penicillin. Particularly has this been true of production on an industrial scale, and although considerable attention and effort have been given to the development of synthetic media for this purpose (Pratt and Dufrenoy, 1945; White *et al.*, 1945; Stone *et al.*, 1945; Foster *et al.*, 1943, 1946), these media have not, apparently, been successfully adapted to large-scale commercial production.

The steady, successive advances made in the search for improved strains of penicillia (Johnson *et al.*, 1946; Backus, Stauffer, and Johnson, 1946) to be used industrially have seemingly eclipsed such advantages of synthetic media as appeared inherent in this type of medium as long as broth potencies were comparatively low. This applies to surface and submerged processes alike. The principal advantage of a synthetic medium lies in its uniformity and reproducibility from time to time, and, in relation to low potency broths, in the fact that in such a medium fewer organic acidic substances are present as impurities than are found in corn steep liquor broth, thus making possible the extraction of a more potent, less toxic, and generally more suitable penicillin product for clinical purposes. Also, improved extraction yields are possible. These advantages have been fairly well overshadowed by improved extraction procedures, by enormous increases in the penicillin content of the medium with essentially the same total amount of impurities, and by the considerably faster rate of penicillin formation in corn steep liquor medium.

The well-known fluctuations from batch to batch of corn steep liquor prompted a search for an effective, but inexpensive natural material which would be available in bulk and which might not have this disadvantage. An extract of ground dried peas at 10 per cent concentration has been reported (Cook *et al.*, 1944) as a successful ingredient of penicillin media, but the feasibility of using this substance in industry seems remote, at least in the United States.

Of a large number and variety of different organic materials tested in submerged culture with *Penicillium notatum* NRRL 832 in this laboratory, corn steep liquor always was outstanding, despite the generally recognized fact that almost all the other complex, natural proteinaceous materials permitted as abundant growth of the fungus as did corn steep liquor without, however, being able to

stimulate penicillin formation. This distinctive property of corn steep liquor has been intensively investigated and is due in part at least to the presence in corn steep liquor of stimulating substances such as the amino acids arginine, histidine, and glutamic acid (White *et al.*, 1945) and possibly to derivatives of phenylacetic acid (Pratt and Dufrenoy, 1945).

As shown in a previous paper of this series (Foster *et al.*, 1946), cottonseed meal, though definitely inferior to corn steep liquor, did nevertheless stimulate penicillin production by strain 832 sufficiently well to mark it as decidedly better than all other materials with which it was compared. The availability of the newer and improved strains, *Penicillium chrysogenum* Demerec X1612 and *P. chrysogenum* Wisconsin Q176, derived by Backus and Stauffer from strain X1612, led to a retest of cottonseed meal in the belief that the behavior of these strains relative to cottonseed meal and corn steep liquor might be different from that of strain 832. This proved to be the case, for with both these strains cottonseed meal was equivalent or superior to corn steep liquor. Table 1 summarizes a comparative experiment. The optimum concentration of corn steep liquor was 2 per cent (solids), and that of cottonseed meal was 4 per cent.

From table 1 the following conclusions are evident: Cottonseed meal is considerably better than corn steep liquor for penicillin production by both strains in the absence of special stimulating chemicals of the phenylacetyl-derivative type. The stimulating effect of phenylacetyl derivatives (Pratt and Dufrenoy, 1945) is small in cottonseed meal medium, whereas it is very marked in corn steep liquor medium, in which it is able to bring the level of penicillin production by corn steep liquor up to a range approximately as high as that of cottonseed meal. Apparently, corn steep liquor is deficient in such factors, whereas they are not limiting in cottonseed meal.

Other conclusions derived from a substantial experience with cottonseed meal are as follows:

(1) All lots of cottonseed meal or whole cottonseeds are approximately equal in penicillin-producing ability. There is some indication that less of the high protein (40 per cent) meal is required than of meal of low protein content (28 per cent). Generally 3 to 4 per cent cottonseed meal is necessary for maximum potency together with 2 to 3 per cent lactose.

(2) CaCO_3 is necessary for maximum activity on laboratory shakers although it may not be necessary in large fermenters.

(3) Activities are somewhat higher with NaNO_3 in the medium.

(4) The pH remains in a favorable range during the whole fermentation period in cottonseed meal media.

(5) In fermenters, less foaming is encountered with cottonseed meal than with corn steep liquor.

(6) In pilot plant fermenters with strain X1612, somewhat higher activities were obtained with 0.5 per cent NaNO_3 , and no CaCO_3 , in cottonseed medium.

(7) Under comparable conditions, with strain X1612, a series of pilot plant fermenter yields averaged about 400 units per ml with cottonseed meal as against 120 with corn steep liquor. In laboratory experiments, 400 units per ml was

the maximum for both media. Also the rate of growth exceeds that in corn steep media.

(8) Both strains require an adaptation to lactose for maximum effectiveness. Thus, strain X1612 inoculum prepared in lactose medium began to consume the lactose in a fermenter 26 hours after inoculation, whereas the inoculum prepared in a medium with glucose instead of lactose began to attack the lactose in the fermenter only after 50 hours, and the penicillin activity reached was substantially lower than with an adapted inoculum.

TABLE 1

*Comparison of corn steep liquor and cottonseed meal with two different strains of *P. chrysogenum***

MEDIUM	DEMEREK STRAIN X1612				WISCONSIN STRAIN Q176			
	3 days	4 days	5 days	6 days	3 days	4 days	5 days	6 days
2 per cent corn steep solids 3 per cent lactose 1 per cent CaCO ₃	140	180	200	120	375	550	650	600
4 per cent cottonseed meal 3 per cent lactose 1 per cent CaCO ₃	190	250	300	175	520	800	950	1,000
Corn steep liquor medium plus 0.05 per cent phenylacetyl derivative	160	250	350	300	1,050	1,300	1,250	900
Cottonseed meal medium plus 0.05 per cent phenylacetyl derivative	160	270	320	200	540	950	1,160	885

* Data represent Oxford units per ml and are averages of triplicate flasks; submerged cultures on rotary shakers; eighty-ml medium per 250-ml Erlenmeyer flask; temp. 23 to 25 C.

SUMMARY

Cottonseed meal is at least as good as corn steep liquor for penicillin production by *Penicillium chrysogenum*, strains Demerec X1612 and Wisconsin Q176. Without added chemical precursors, cottonseed meal is considerably superior to corn steep liquor. A number of the characteristics of the cottonseed meal medium are discussed. *P. chrysogenum* required an adaptation to lactose for the most rapid and efficient utilization of lactose in laboratory fermenters.

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MUTATION IN CERTAIN PHYTOPATHOGENIC BACTERIA INDUCED BY ACENAPHTHENE

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Mutation in higher plants and fungi is a well-recognized phenomenon. Its artificial induction may be accomplished by various means which are important in obtaining new varieties of useful plants and in the prosecution of mycological and genetical problems.

Blakeslee and Avery (1937) produced mutants in plants by means of an alkaloid of the phenanthrene series, colchicine, which occurs in a high concentration in *Colchicum autumnale*. It is a mitotic poison capable of doubling the number of chromosomes and thus inducing polyploidy. However, attempts to induce changes in fungi and bacteria by means of colchicine have met with failure (Blakeslee, 1939). Jennison (1940) studied the effects of 1 per cent colchicine in nutrient media on ten species of pathogenic bacteria, with no indication of either morphological or physiological changes. Similar results were also obtained by Bonetti and Illenyi (1941). Walker and Youmans (1940) found that 1 per cent colchicine inhibited the growth of *Streptococcus hemolyticus*, but 0.5 per cent and 0.25 per cent had no effect. *Staphylococcus aureus* H developed a somewhat different type of growth in 2 per cent colchicine broth. On solid media wrinkled, waxy, and peculiarly greenish-yellow colonies developed, which obtained only in the presence of colchicine. They reverted to normal type immediately after being transferred to plain media. Thus it appears that colchicine does not disturb the structural apparatus of bacterial cells. In this connection it is interesting to note that colchicine does not affect the cells of *Colchicum autumnale*, from which it is derived (Levan, 1940).

In recent years a chemical of the cholanthrene group, known as acenaphthene, $C_{16}H_8(CH_2)_2$, has been found to cause profound polyploidy in plants (Shmuck, 1938). Extensive studies of this compound indicate that in some respects it may be superior to colchicine in throwing light upon the dynamics of cell division in general and meiosis in particular (Kostoff, 1938a, 1938b; Navashin, 1938). It has been determined that 7 μ g of acenaphthene will cause a profound disturbance in one wheat seed with the formation of a polyploid set of chromosomes (Shmuck and Gusseva, 1939a).

METHODS AND RESULTS

The writer has found that acenaphthene induced permanent changes in certain phytopathogenic bacteria. Mutation was induced in *Phytophthora michiganensis* and *Erwinia carotovora* by growing them in nutrient broth saturated with acenaphthene, a medium which was unchanged by previous autoclaving at 15 pounds' pressure for 10 minutes. The inoculum was grown in liquid media, and

only very young cultures were used in the experiments. It was found that the bacteria used in these experiments mutated most readily if recently isolated from their respective hosts. In some cells mutations occurred after two weeks' growth in acenaphthene broth at 28 C. The mutants were detected on agar plates by dilution or streaking. Acenaphthene used in solid media failed to induce any mutation in either form.

Phytomonas michiganensis inoculated into acenaphthene broth at first showed some lag in the rate of growth but soon grew as well as in nutrient broth minus acenaphthene. Apparently a sudden and complete mutation occurs in *P. michiganensis*, since neither intermediate nor typical forms could be found in acenaphthene broth after a certain time, and only a white, slimy, smooth type of colony developed on agar plates. These forms were somewhat more virulent than the parent culture but otherwise have remained similar to the parent, except in colony type, for the last five years under laboratory conditions. There was no deviation from the parent culture so far as sugar fermentation and other reactions were concerned. Morphologically both types were alike. Previously, a similar white strain of *P. michiganensis* had been obtained as a variant in old cultures as well as from field material (Fawcett and Bryan, 1934; Ark, 1944).

Occasionally, when inoculated tubes of the acephthene series were left for some time, a very slow-growing, white, flat type of colony became evident. This proved to be another mutant of *Phytomonas michiganensis*, which was very difficult to maintain in the laboratory and showed no pathogenicity to tomato plants. Unsuccessful attempts were made to change the white race of *P. michiganensis* into the yellow (parental) form by the acenaphthene method.

A recent isolate of *Erwinia carotovora*, from soft rot of carrot, responded to the acenaphthene treatment by producing a mutant. However, a mixture of types prevailed, i.e., normal as well as mutated forms were present when the plates were prepared from 2-week or older acenaphthene broth cultures of the organism. The prevailing type was a grayish-white, compact, flat colony which grew slower than the parent strain. It was only slightly pathogenic on carrot slabs. No physiological or morphological differences were noticeable in common laboratory media.

Phytomonas phaseoli, a yellow pathogen, did not produce any mutants in acenaphthene broth even after very prolonged growth therein.

Thus, acenaphthene does not seem to have similar effects on all species of bacteria. This indication is in agreement with the findings of Shmuck and Gusseva (1939b), who found that acenaphthene was not biologically active for the leguminous plants, but strongly so for the cereals.

SUMMARY

Acenaphthene-saturated broth is capable of inducing permanent mutations in *Phytomonas michiganensis* and *Erwinia carotovora*.

Some mutants of *Erwinia carotovora* possessed a reduced pathogenicity for carrot roots, and one mutant of *Phytomonas michiganensis* was nonpathogenic.

Acenaphthene does not act similarly on all species of bacteria.

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A METHOD FOR THE DETERMINATION OF THE CULTURE CYCLE AND THE GROWTH RATE OF VIRULENT HUMAN TYPE TUBERCLE BACILLI¹

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Accurate determinations of the normal culture cycle and the growth rate of *Mycobacterium tuberculosis* var. *hominis*, to the knowledge of the author, have never been reported. Until recent years this organism has routinely been cultivated in the form of a surface pellicle on solid or liquid media. Under these conditions tubercle bacilli grow in relatively dry coherent masses, and neither direct counts nor plate counts can be used for an estimation of the number of organisms present. Attempts have been made to measure the amount and rate of growth of tubercle bacilli by drying and weighing the total surface pellicle growth from liquid media after varying intervals of time. The technical difficulties and the inaccuracies of this method for the determination of bacterial mass have been pointed out by Mueller (1935). Furthermore, when tubercle bacilli are employed, this method is even more inaccurate since a uniform inoculum cannot be used and the rate and type of growth may vary from flask to flask. In addition, since relatively large amounts of growth are necessary for the determinations, the earlier periods of the growth cycle might well be missed.

Accurate determination of the growth rate would be of value for the determination of the effect of physical or chemical agents on the growth of virulent tubercle bacilli. Previously such information either has been obtained subjectively, with a consequent large and unpredictable degree of error, or has been determined by the difference in total mass of culture after an arbitrary growth period. The latter method, however, frequently gives values that bear no relation to the rate of growth.

The demonstration by Drea (1940, 1942) and Youmans (1944a, 1944b) that fine suspensions of virulent human type tubercle bacilli will grow readily beneath the surface of synthetic media permits the use of a uniform homogeneous inoculum and leaves only the problem of measuring accurately the subsequent growth. Since nitrogen is a relatively uniform constituent of living cells, Mueller (1935) recommended the use of nitrogen determinations on cultures of bacteria for determining the amount of growth. Hershey (1939) has shown that nitrogen determinations on growing cultures of *Escherichia coli* give values that are proportional to the total mass of growth, although not always proportional to the number of organisms present.

The present paper details the use of micro-Kjeldahl nitrogen determinations for the purpose of determining the normal culture cycle, the growth rate, and the generation time of the H37Rv strain of virulent human type tubercle bacilli.

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METHODS

The synthetic medium employed contained asparagine, 0.5 per cent; mono-potassium phosphate, 0.5 per cent; magnesium citrate, 0.15 per cent; potassium sulfate, 0.05 per cent; and glycerol, 2.0 per cent; dissolved in water redistilled from glass. The pH was adjusted to 7.0 with sodium hydroxide, using a Coleman electrometer, and the medium was sterilized in the autoclave at 10 pounds' pressure for 20 minutes, although higher temperatures can be used without ill effect.

Pyrex test tubes, 200 by 25 mm, were cleaned by standing overnight in concentrated sulfuric acid. They were then thoroughly rinsed with tap water and distilled water and allowed to dry. When dry they were plugged with cotton and sterilized in the autoclave at 20 pounds' pressure for 20 minutes. Following sterilization, 10.0 ml of sterile synthetic medium were introduced aseptically into each tube using an accurately calibrated volumetric pipette.

The entire surface pellicle growth of a 21-day-old flask culture of the H37Rv strain of *M. tuberculosis* was washed by centrifugation three times with 20- to 30-ml portions of sterile 0.01 molar phosphate buffer solution, pH 7.0. The washed tubercle bacilli were transferred to a sterile mortar and ground with a sterile pestle, with the gradual addition of 0.01 molar phosphate buffer solution until a relatively homogeneous suspension resulted. Following transfer to a sterile tube the suspension was allowed to stand for 30 minutes to permit the larger clumps to settle out. The fine supernatant suspension was transferred to a sterile tube and standardized by micro-Kjeldahl determinations on suitable aliquot portions. Before the nitrogen determinations were done it was necessary to centrifuge and wash the aliquot portions in the manner to be described below, because in suspensions prepared in this fashion 30 to 40 per cent of the nitrogen was found to be in solution, possibly due to the rupture of many organisms during grinding.

Following standardization, the suspension was diluted with 0.01 molar phosphate buffer solution to the desired nitrogen concentration, and each tube of medium was inoculated with 1.0 ml of this diluted suspension, using a volumetric pipette. The tubes were shaken, capped with waxed paper, and incubated at 37 C.

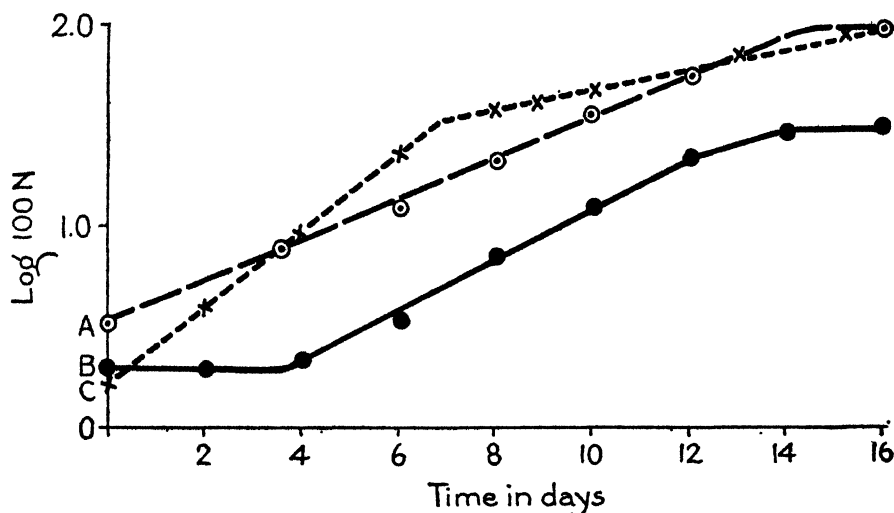
At intervals following inoculation, usually every 2 days, a number of tubes were removed from the incubator and heated in a boiling water bath for 10 minutes; to each tube was then added, at room temperature, 10.0 ml of a saturated solution of potassium lauryl sulfate, and 1.0 ml of a 2 per cent suspension of super-cel. The tubes were centrifuged at about 3,000 rpm for 5 minutes, and the supernatant fluid was removed from the sediment by decantation or aspiration. The organisms were then washed twice with 20.0-ml portions of the potassium lauryl sulfate solution. After the final washing, digestion and nitrogen determinations of the entire contents of each tube were made according to the method of Ma and Zuazaga (1942).

Numerous experiments have shown that the preliminary heating in this procedure did not alter the nitrogen content. The potassium lauryl sulfate solution served as a wetting agent and prevented the fine growth of tubercle bacilli from climbing the sides of the tubes or from floating on the surface of the liquid. The

super-cel greatly facilitated centrifugation, giving a more firmly packed sediment, and greatly reduced the centrifugation time.

RESULTS

Three types of growth curve were obtained with the virulent human type tubercle bacillus, strain H37Rv, and are illustrated in graph 1. Curve B is similar to those obtained with other bacteria when the plate count method is employed: a lag phase followed by a period of logarithmic growth and finally by a period of decreasing growth. Curves A and C, however, show that tubercle bacilli may begin immediately to grow at a constant rate. According to Hershey (1939) and Winslow and Walker (1939) in experiments with *E. coli*, when determinations of cell mass were made using a favorable medium for growth of the culture, there



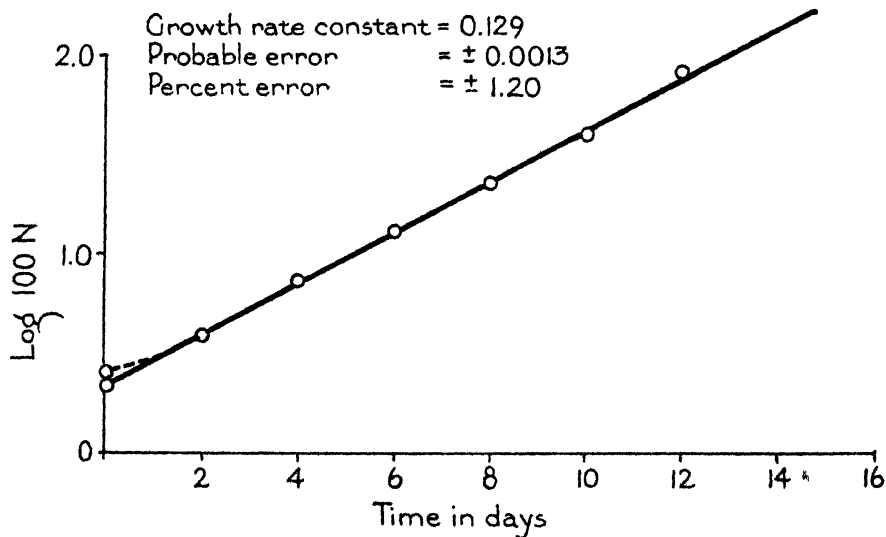
GRAPH 1. NORMAL CULTURE CYCLES OF *M. TUBERCULOSIS* (H37Rv)

was no lag phase regardless of the age of the culture used for inoculation. The logarithmic growth phase started immediately because of the fact that the total mass of the culture increased at a constant rate even though cell division was retarded. The synthetic medium used for the tubercle bacilli would theoretically be a relatively unfavorable one; therefore, it was of interest that occasionally no evidence of a lag phase was obtained. It is possible, of course, that the results in graph 1 might have been due to the fact that the organisms used as an inoculum had, in the case of curve B, already passed the period of logarithmic growth, whereas those used in curves A and C were in the logarithmic growth phase at the time of inoculation. Curve C is also interesting since we have on several occasions noted this very rapid rate of growth for a period of 6 to 8 days, followed by a sudden decrease in rate, which then remained constant for 6 to 8 days. No obvious explanation for this phenomenon presents itself, since all known physical and chemical factors were carefully controlled.

In table 1 are shown the growth rate constants and the generation times of ten separate growth curve determinations obtained by using 21-day-old surface cultures of H37Rv as inocula. The values in the table were obtained by plotting the logarithms of the nitrogen values against the time in days and drawing the

TABLE 1
Growth rate constants and generation times of *M. tuberculosis* (H37Rv)

	AMT. OF INOCULUM IN MG OF N	GROWTH RATE CONSTANTS	GENERATION TIME IN DAYS
1	0.04	0.08	3.70
2	0.085	0.10	3.01
3	0.025	0.11	2.73
4	0.02	0.125	2.48
5	0.03	0.13	2.31
6	0.015	0.13	2.31
7	0.035	0.14	2.13
8	0.02	0.15	2.00
9	0.03	0.185	1.62
10	0.015	0.195	1.54

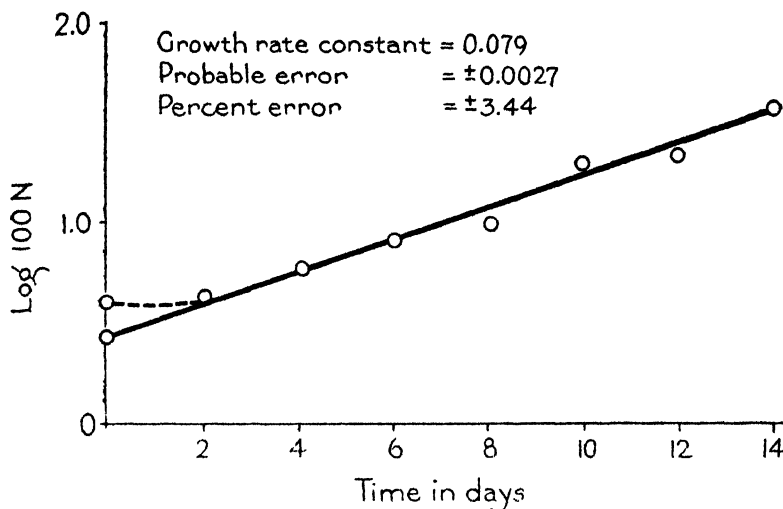


GRAPH 2

curves that best fitted the data. The growth curve constants and the generation times were calculated from the straight-line portions of the curves, the logarithmic growth phase. In most cases the slope of the line was determined by inspection, but two curves were selected which seemed to represent, respectively, experiments in which the points deviated least markedly and most markedly from a straight line, and the rate of growth and the probable error were calculated by the method of least squares. The probable errors were, respectively, plus or

minus 1.2 and 3.4 per cent. These are illustrated in graphs 2 and 3. In graph 2 six tubes of growth were used to determine each point on the curve, and in graph 3 three tubes were used. Therefore it would appear that, depending on the number of tubes of growth analyzed and the amount of care exercised in the determinations, the growth rate determinations have an accuracy of between a plus or minus 5 to 15 per cent.

As shown by the data in table 1, the growth rates, and therefore the generation times, varied markedly regardless of the amount of inoculum or the age of the culture used for inoculation. The chemical composition of the medium and the physical growth conditions were constant; consequently, these results would seem to represent differences in the physiological state of the cultures used as inocula.



GRAPH 3

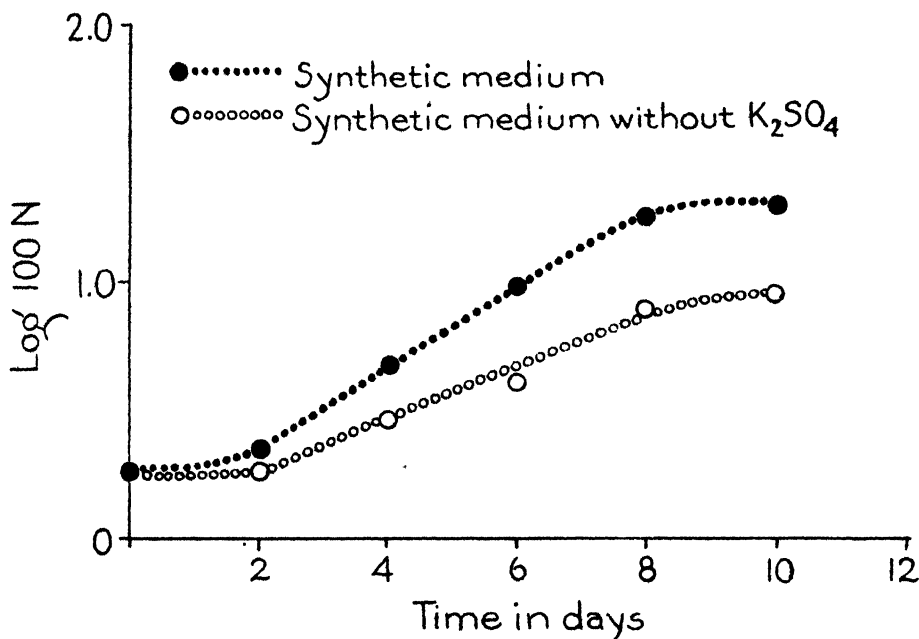
Similar divergent results have been obtained in a few experiments using 14-day-old cultures as inocula.

It should also be pointed out that in these experiments measurements are first made of the amount of subsurface growth and secondly of combined subsurface and surface growth since, depending on the amount of inoculum and the rate of growth, a surface pellicle will begin to form in the culture tubes in from 4 to 8 days.

Graph 4 shows the effect on the rate and amount of growth of the H37Rv strain when the potassium sulfate was omitted from the synthetic medium. The sulfur requirements of the tubercle bacillus are not well known, but the results show that although growth occurs in its absence, except for such small amounts as may be present as impurities in the other constituents of the medium, it is considerably reduced in rate and amount.

The usefulness of the method for the determination of the degree of inhibition of growth produced by bacteriostatic substances is illustrated in graphs 5 and 6. The substance used, 4,4' diamino diphenyl sulfone, exerts a suppressive effect on

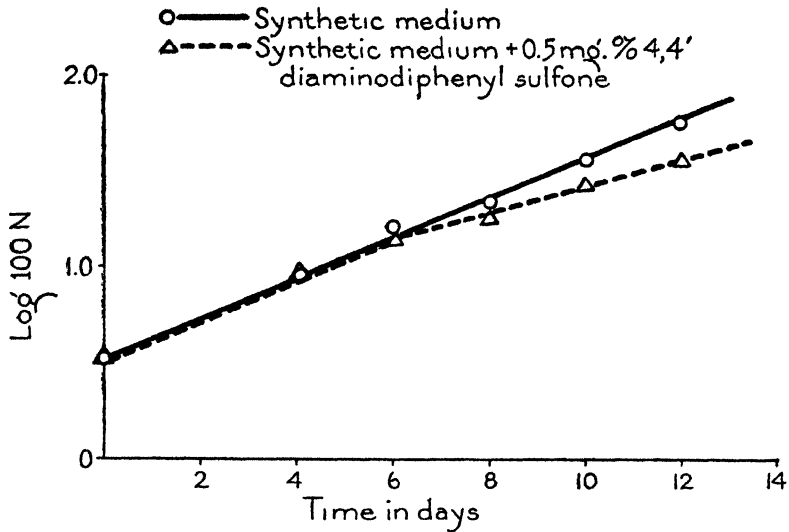
experimental tuberculosis of guinea pigs (Feldman, Hinshaw, and Moses, 1943), and derivatives of this compound have had clinical trial as chemotherapeutic agents for tuberculosis of humans. This compound has also been shown to be bacteriostatic for tubercle bacilli *in vitro* (Steenken and Heise, 1943; Youmans, 1944b). When 0.05 mg per cent of 4,4' diamino diphenyl sulfone was present in the medium, the rate of growth was inhibited approximately 28 per cent (graph 5). With a concentration of 1.0 mg per cent, the inhibition of the rate



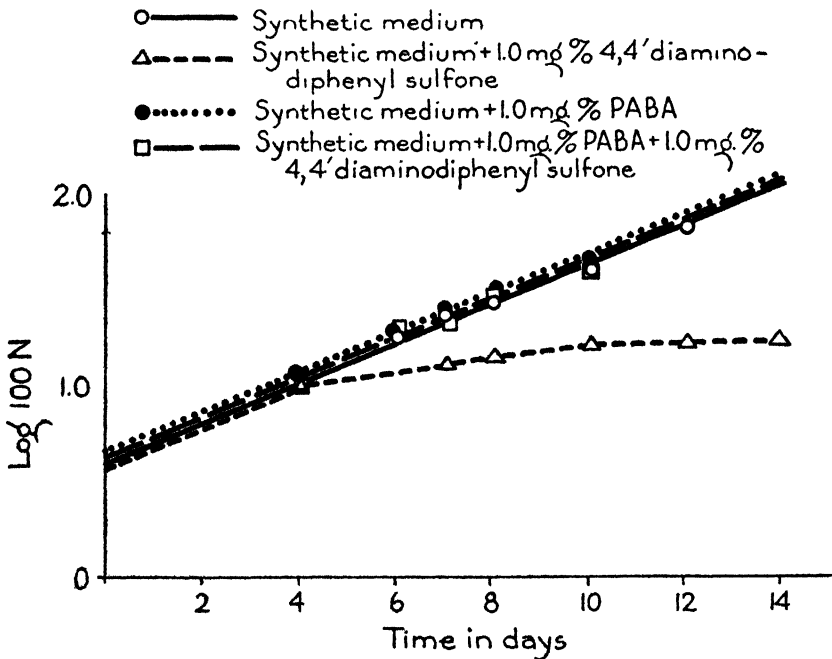
GRAPH 4. EFFECT OF OMISSION OF K_2SO_4 FROM SYNTHETIC MEDIUM ON GROWTH OF *M. TUBERCULOSIS* (H37Rv)

of growth was approximately 62 per cent. Furthermore, 1.0 mg per cent *para*-aminobenzoic acid completely reversed the bacteriostatic action of 4,4' diamino diphenyl sulfone and did not itself exert any stimulating or inhibitory effect on the growth of the tubercle bacilli (graph 6). Of further interest is the fact that the method clearly shows a lag in the inhibitory action of this sulfone on the tubercle bacillus similar to the lag in the action of the sulfonamides that has been observed with other bacteria (Henry, 1943).

The validity of this method for the determination of the amount and rate of growth of tubercle bacilli is, of course, based on the assumption that the increase in bacterial nitrogen is proportional to the increase in the total mass of the bacterial population. This has been shown to be true of *E. coli* (Hershey, 1939), and presumably would also be true of the tubercle bacillus. However, the possibility should be kept in mind that under conditions different from those used in the present work the ratio of nitrogen to the total mass might be different.



GRAPH 5. THE EFFECT OF 0.5 MG PER CENT 4,4' DIAMINO DIPHENYL SULFONE ON GROWTH OF *M. TUBERCULOSIS* (H37Rv)



GRAPH 6. THE EFFECT OF 1.0 MG PER CENT 4,4' DIAMINO DIPHENYL SULFONE AND PABA ON GROWTH OF *M. TUBERCULOSIS* (H37Rv)

SUMMARY

By the use of micro-Kjeldahl nitrogen determinations, the culture cycle of the virulent human type tubercle bacillus, strain H37Rv, was determined. From the logarithmic portion of the growth curve, the growth rates and generation times were calculated. Under the conditions of the experiment the generation times were found to vary between approximately $1\frac{1}{2}$ and $3\frac{1}{2}$ days. The usefulness of the method for accurately determining the amount of growth of tubercle bacilli and the effect on the rate of growth of alteration of the composition of the medium and of the presence of growth-inhibiting substances is illustrated.

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SYNTHESIS OF A POLYSACCHARIDE FROM SUCROSE BY *STREPTOCOCCUS S.B.E.*

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The identity of 113 cultures of streptococci recovered from cases of subacute bacterial endocarditis has been reported recently (Niven and White, 1946). Of this number, 42 seemed to represent a hitherto unrecognized variety, or species, which has been tentatively labeled *Streptococcus s.b.e.* In contrast with other streptococci associated with endocarditis those cases caused by *Streptococcus s.b.e.* respond sluggishly, or not at all, to Loewe's penicillin-anticoagulant therapy (Loewe, 1945a, 1945b; Loewe, Plummer, Niven, and Sherman, 1946).

Since prompt identification of *Streptococcus s.b.e.* may be a lifesaving measure, adequate methods for accomplishing this would be desirable. A detailed description of this streptococcus is to be given in a forthcoming publication. In the present report a unique physiological characteristic of *Streptococcus s.b.e.* will be described, namely, the ability to synthesize a polysaccharide from sucrose in broth culture.

METHODS AND RESULTS

In determining the fermentation characteristics of our collection of streptococci from endocarditis (White, 1944) it was noticed that some of the cultures when grown in a 1 per cent sucrose broth appeared to become slightly viscous. Practically all strains having this property were identified as *Streptococcus s.b.e.* In line with this suggestion, a large part of the collection has been restudied with respect to polysaccharide synthesis from sucrose.

The ability to form large, mucoid colonies on a 5 per cent sucrose agar (Niven, Smiley, and Sherman, 1941a) has become a convenient and accurate presumptive test for the identification of *Streptococcus salivarius*. As opposed to the picture presented by *Streptococcus salivarius*, none of the *Streptococcus s.b.e.* showed any evidence of mucoid colonies when streak cultures were observed on this medium after 24 hours' incubation at 37 C. However, after the incubation was continued for 3 additional days, 24 cultures showed slight evidence of polysaccharide synthesis. The individual colonies appeared very much like minute, clear-glass beads scattered over the surface of the plate. On the thickly seeded portion of the plate the mucoid appearance was somewhat more evident. The mucoid appearance upon the plate culture, however, could be easily missed by a casual observer. Eight of the *Streptococcus s.b.e.* cultures showed no evidence of polysaccharide synthesis upon continued incubation.

The cultures were then tested in a sucrose broth having the following composition: 1 per cent tryptone, 0.5 per cent yeast extract, 0.5 per cent K_2HPO_4 , and 5.0 per cent sucrose; pH 7.4. The results obtained in this medium were striking.

After an incubation period of 3 days at 37 C, 31 of the 34 *Streptococcus s.b.e.* cultures tested showed a marked increase in the viscosity of the broth (table 1). Nine of the cultures actually solidified the medium, these cultures having the consistency of about a 1 per cent agar. The viscous nature of those cultures which did not solidify the medium could be easily determined by agitating the contents of the tubes so as to incorporate a few small air bubbles. When the agitation ceased, there was an immediate arrest in the motion of the bubbles and the suspension of bacterial cells. Upon continued incubation, there was little or no tendency for the cells to settle out of the sucrose medium, whereas the same strains grown in a 5 per cent glucose broth would tend to settle out leaving a clear supernatant.

The three strains which failed to synthesize the polysaccharide grew poorly in the sucrose medium. It is assumed that the reason for failure with these cultures was an insufficient medium for optimum growth. These strains, two of which had been isolated from one patient, had always grown poorly in laboratory media. When tested again in a medium containing only 3 per cent sucrose and fortified

TABLE 1
Polysaccharide synthesis from sucrose by endocarditis cultures

SPECIES OR VARIETY	CULTURES TESTED	CULTURES SHOWING CARBOHYDRATE SYNTHESIS	
		Sucrose agar streaks	Sucrose broth
<i>Streptococcus s.b.e.</i>	34	24 (slight)	32
<i>Streptococcus mitis</i>	29	0	2
<i>Streptococcus bovis</i>	7	7	7
Miscellaneous.....	18	0	0

with beef infusion, one of the three strains showed marked evidence of polysaccharide synthesis.

Among the collection of streptococci tested in the sucrose broth were seven cultures which had been previously identified as *Streptococcus bovis*. All these strains produced mucoid colonies on sucrose agar similar to certain members of this species from bovine sources (Niven, Smiley, and Sherman, 1941b). In the 5 per cent sucrose broth these cultures also synthesized a polysaccharide as evidenced by a marked increase in the turbidity of the culture medium, with a slight increase in viscosity. Only one strain, however, increased the viscosity to such an extent that it could not be distinguished from *Streptococcus s.b.e.* The *Streptococcus bovis* cultures from endocarditis were of the "indifferent" type on blood agar, in addition to having several other physiological characteristics which would afford easy differentiation from *Streptococcus s.b.e.*

Of the 29 strains of *Streptococcus mitis* cultures from endocarditis tested, none showed any evidence of mucoid colony production on sucrose agar, but two strains were found which appeared to be identical to *Streptococcus s.b.e.* in sucrose broth. In contrast to *Streptococcus s.b.e.*, these strains fermented raffinose, but

not inulin, and failed to produce ammonia from arginine. They could also be differentiated from *Streptococcus* s.b.e. by serological methods.

Of the remaining 18 streptococcus cultures from endocarditis tested, none was found to show any evidence of polysaccharide synthesis from sucrose, either on the agar plate or in broth culture. In this group were cultures which had been identified as *Streptococcus agalactiae*, *Streptococcus faecalis*, Lancefield group G (non-minute variety), and four strains which could not be identified by either physiological or serological methods.

POLYSACCHARIDE SYNTHESIS FROM OTHER SUGARS

Three strains of *Streptococcus* s.b.e., one *Streptococcus bovis*, and the two polysaccharide-synthesizing cultures of *Streptococcus mitis* were inoculated into broth media containing 5 per cent each of all the various sugars, polysaccharides, and higher alcohols commonly used in fermentation tests. Also included was a broth containing both glucose and fructose at a level of 2.5 per cent each. There was no evidence of polysaccharide synthesis by any of the strains on any of the test substances except sucrose.

Of interest is the fact that none of the strains synthesized visual quantities of polysaccharide from raffinose. Although *Streptococcus* s.b.e. does not characteristically ferment this sugar, one strain was included which possessed this property. The three *Streptococcus bovis* and the two *Streptococcus mitis* cultures included were able to ferment raffinose. In contrast to these findings, all *Streptococcus salivarius* strains from the human throat are able to synthesize a levan from both sucrose and raffinose (Niven, Smiley, and Sherman, 1941a).

One strain of *Streptococcus* s.b.e. was tested for its ability to synthesize a polysaccharide from sucrose in cell suspension. The cells were grown for 18 hours in 0.3 per cent sucrose broth, then centrifuged out, washed once, and concentrated tenfold in a phosphate-buffered, 5 per cent sucrose solution, pH 7.4. The suspension was incubated at 37 C and neutralized occasionally with strong NaOH. Within 8 hours the contents of the flask had solidified.

PHYSICAL AND CHEMICAL PROPERTIES OF THE POLYSACCHARIDE

Two liters of the 5 per cent sucrose medium were inoculated with a strain of *Streptococcus* s.b.e. and incubated at 37 C. At occasional intervals the acid produced was neutralized with strong NaOH. When acid production ceased (in 48 hours), the flask was steamed for 30 minutes to kill the cells. Ethyl alcohol was added to the semisolid medium, with vigorous agitation, to approximately 50 per cent concentration. At this stage a large quantity of gelatinous precipitate settled out of the medium. The precipitate was washed several times in 50 per cent alcohol and then resuspended in water. The precipitation procedure was repeated three times, after which the precipitate was suspended in water and dialyzed for 24 hours against running tap water.

The suspension was then precipitated with excess alcohol, washed twice with 95 per cent alcohol, and then spread out to dry for 24 hours at 110 C. Even though no attempts were made to recover the polysaccharide quantitatively,

21 grams of the dried substance were obtained from the 160 grams of added sucrose in the original medium.

This dried substance appeared to be a mixture of two polysaccharides, one fraction (estimated to be about 10 per cent) being soluble in water. The water-soluble component was removed by suspending the dried material in water with mechanical stirring, followed by centrifuging. The operation was repeated until no alcohol-precipitable material was found in the supernatant. No attempts were made to identify the water-soluble fraction.

Even though it is insoluble in water, the larger fraction is characterized by its marked ability to imbibe water, resulting in a viscous suspension at a 1 per cent concentration. It can be centrifuged out easily to a thick, gelatinous mass, or can be precipitated out with 50 per cent alcohol. In normal HCl it goes into a turbid colloidal suspension with loss in viscosity. It is soluble in normal NaOH. There was found to be 0.1 per cent nitrogen and 0.5 per cent ash in the purified and dried material.

The polysaccharide was difficult to hydrolyze. A 2 per cent suspension in normal HCl heated for 4 hours at 100 C did not result in complete hydrolysis, as judged by the rate of increase in reducing the sugar content of aliquots taken at 30-minute intervals. At the end of the heating period 83 per cent of the carbohydrate could be accounted for as reducing sugars (calculated as glucose). The optical activity of the clarified hydrolyzate indicated 84 per cent glucose. Therefore, it was tentatively concluded that the insoluble polysaccharide produced by the *Streptococcus* s.b.e. strain was a dextran.

DISCUSSION

As this report was being prepared, our attention was called to a recent article by Hehre and Neill (1946) concerning the production of a dextran from sucrose by 22 of their 45 cultures of streptococci isolated from cases of subacute bacterial endocarditis. From the description given by the authors it would appear highly probable that most, if not all, of their dextran-producing cultures are identical with *Streptococcus* s.b.e. Our tentative identification of the polysaccharide as a dextran is in entire conformity with their results employing serological methods.

An interesting observation reported by Hehre and Neill is that, although very little or no dextran is synthesized by their cultures on sucrose agar incubated aerobically, all strains produced mucoid colonies on the same agar under anaerobic conditions. We have not tested our cultures in this manner.

Assuming that the cultures of Hehre and Neill are identical with *Streptococcus* s.b.e., it is interesting to note that this organism has been found to occur in a large proportion of the cases of endocarditis, as determined independently in two different laboratories. As will be pointed out in a future publication, this streptococcus (with one exception) has not been encountered from sources other than cases of endocarditis.

Because of the relatively large proportion of fatal cases due to this organism, it might at times be highly desirable to recognize this organism during the early

stages of the disease. The production of a dextran in sucrose broth (or perhaps on sucrose agar plates incubated anaerobically) might prove to be a valuable presumptive test.

Although *Streptococcus salivarius* was not encountered in our collection of endocarditis cultures (nor were there any in the collection of Hehre and Neill), it may at times be necessary to differentiate between these two groups of streptococci. Although there are many other physiological differences which could be used, this can be accomplished easily by merely observing the types of colonies on sucrose agar and the changes taking place in sucrose broth. *Streptococcus salivarius* produces large mucoid colonies on sucrose agar within 24 hours (aerobically), and in sucrose broth a bluish opalescence is developed in the supernatant with no apparent increase in viscosity. Some cultures of *Streptococcus salivarius* also synthesize an insoluble dextran from sucrose (Niven, Smiley, and Sherman, 1941b), but this material settles to the bottom of the tube in a flocculent mass along with the bulk of the bacterial cells.

Since a few cultures of *Streptococcus bovis* and *Streptococcus mitis* which produce a viscous dextran in sucrose broth may also be encountered in endocarditis cases, this test cannot be considered a perfect one. Other physiological or serological procedures would also have to be used in order to identify *Streptococcus* s.b.e. positively.

Since some strains of *Streptococcus* s.b.e. solidify the sucrose broth, whereas others merely increase the viscosity of the medium, it might be concluded that these organisms are synthesizing different types of polysaccharides. It is felt, however, that this is merely a quantitative effect; those strains which solidify the broth may be synthesizing larger quantities of the same polysaccharide.

SUMMARY

Thirty-two of the 34 strains of *Streptococcus* s.b.e. recovered from cases of subacute bacterial endocarditis synthesized large quantities of a polysaccharide from sucrose in broth culture, as determined by an increase in viscosity or by actual solidification of the medium. These cultures synthesized little or no polysaccharide when streaked on sucrose agar.

This unique property may be helpful in the identification of *Streptococcus* s.b.e., such cultures having been recovered from approximately one-third of the cases studied.

The polysaccharide has been tentatively identified as a dextran.

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STREPTOCOCCUS S.B.E.: A STREPTOCOCCUS ASSOCIATED WITH SUBACUTE BACTERIAL ENDOCARDITIS

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In two preliminary publications (Loewe, Plummer, Niven, and Sherman, 1946; Niven and White, 1946) a report has been presented outlining the identities of various streptococcus cultures isolated from a large number of cases of subacute bacterial endocarditis. One of the largest groups recorded appeared to represent a hitherto unrecognized variety, or species. This streptococcus, which was recovered from approximately one-third of the patients afflicted with bacterial endocarditis, has several unique physiological characteristics which afford a relatively simple and accurate means for identification. Furthermore, all the strains which have been studied thus far have fallen into one of two serological types. For the sake of convenience this streptococcus is referred to as "*Streptococcus s.b.e.*"

The successful use of penicillin-anticoagulant therapy for subacute bacterial endocarditis by Loewe (1945a, 1945b) has recently received wide attention. For the first time a large proportion of the patients suffering from this disease are being cured. As reported by Loewe, a majority of the cases which do not respond to the treatment are ones from which *Streptococcus s.b.e.* have been recovered.

The purpose of the present publication is to describe in detail the physiological characteristics of *Streptococcus s.b.e.*

SOURCES OF CULTURES

Streptococci recovered from the blood stream or heart vegetation of endocarditis cases have been kindly furnished from seven different hospitals widely distributed over the country. The relative frequency of *Streptococcus s.b.e.* among the cultures from each of these sources was practically the same. To date, 42 cultures of this organism from 36 cases of subacute endocarditis have been studied. In a few instances duplicate cultures were received, one of which had been isolated from the patient's blood or heart vegetation after death, a few weeks after the original blood culture had been taken. In all instances of this kind the duplicate cultures were found to be identical.

PHYSIOLOGICAL CHARACTERISTICS

As the cultures were received, they were examined microscopically for purity; all cultures about which there was any doubt as to purity were reisolated. All cultures were tested for the presence of the enzyme catalase, with entirely negative results. None produced detectable quantities of gas from the fermentation of glucose, and none was found to be bile-soluble.

Several, but not all, cultures of *Streptococcus s.b.e.* were tested on the various Lancefield sera representing groups A through G, with entirely negative results.

The various physiological tests used throughout this study were those described by Sherman (1937), plus a few others which had been developed in this laboratory since that date. Table 1 presents a summary of the general characteristics of this group of streptococci, along with the percentage of cultures deviating from the typical physiological pattern. These cultures were tested as they were received, such work extending over a period of three years.

TABLE 1
Physiological characteristics of Streptococcus s.b.e.

	TYPICAL CHARACTERISTICS	PER CENT HAVING TYPICAL CHARACTERISTICS
Greening of blood agar.....	+	98
Growth at 10 C.....	—	100
Growth at 45 C.....	±	55 (—)
Growth on 40% bile blood agar.....	+	72
Growth in 6.5% sodium chloride.....	—	100
Strong reducing action.....	—	100
Final pH in glucose broth.....	4.6-5.0	100
Arginine hydrolyzed.....	+	100
Sodium hippurate hydrolyzed.....	—	100
Starch hydrolyzed.....	—	72
Slime synthesis, 5% sucrose broth.....	+	95
Arabinose.....	—	100
Xylose.....	—	100
Maltose.....	+	100
Lactose.....	+	100
Sucrose.....	+	100
Trehalose.....	+	98
Raffinose.....	—	67
Inulin.....	+	81
Glycerol.....	—	100
Mannitol.....	—	100
Sorbitol.....	—	98
Salicin.....	+	100
Esculin.....	+	86

Streptococcus s.b.e. is a "viridans" type of streptococcus, generally producing a greening reaction on blood agar. However, one exception was encountered, this culture producing a complete but narrow hemolytic zone after 48 hours' incubation. In conformity with the "viridans" group as defined by Sherman (1937), none of the cultures was capable of initiating growth at 10 C; however, approximately half of them were unable to grow at 45 C.

Of possible significance is the fact that 30 of the 42 cultures were able to grow on blood agar containing 40 per cent bile. The two recognized species of viridans streptococci from human sources, *Streptococcus salivarius* and *Streptococcus mitis*, do not possess this ability.

None of the strains was able to grow in a broth containing 6.5 per cent sodium

chloride, nor did any show strong reducing action in litmus milk. These tests, along with the temperature limits of growth, would adequately differentiate *Streptococcus* s.b.e. from the enterococci.

None of the cultures was able to hydrolyze sodium hippurate. Twelve strains hydrolyzed starch. The final pH produced in a 1 per cent glucose broth varied between 4.6 and 5.0, with an average of 4.8.

A test of primary importance for the identification of *Streptococcus* s.b.e. is the hydrolysis of arginine. The established species of the viridans group of streptococci, namely, *Streptococcus bovis*, *Streptococcus equinus*, *Streptococcus thermophilus*, and *Streptococcus salivarius*, characteristically do not attack this amino acid. All *Streptococcus* s.b.e. cultures in our collection were able to do so. The most likely "species" to cause confusion in this respect is that heterogeneous group of streptococci occurring in the normal human throat classified as *Streptococcus mitis*. About one-third of these organisms hydrolyze arginine (Sherman, Niven, and Smiley, 1943). Of the 45 *Streptococcus mitis* cultures we have studied from endocarditis, 12 hydrolyzed arginine, but other physiological tests offered adequate differentiation between these strains and *Streptococcus* s.b.e.

It has been reported (White, 1944; Niven, Kiziuta, and White, 1946) that *Streptococcus* s.b.e. cultures have the ability to synthesize large amounts of a dextran in broth containing 5 per cent sucrose, but not on sucrose agar. All but two of the cultures tested possessed this ability. The sucrose cultures presented striking appearances, becoming extremely viscous after 24 hours' incubation, and many cultures actually became solidified upon further incubation. This simple test has proved to be of great assistance in identifying *Streptococcus* s.b.e. It is not a perfect one, however. Two cultures of *Streptococcus mitis* and seven *Streptococcus bovis* strains isolated from subacute endocarditis have also been found to synthesize a polysaccharide from sucrose in broth culture, some of these appearing very much like the *Streptococcus* s.b.e. strains.

Recently Hehre and Neill (1946) have reported that certain cultures of streptococci associated with subacute bacterial endocarditis synthesized large amounts of a dextran in sucrose broth, or on sucrose agar plates incubated anaerobically. From the description given by these authors, there would appear to be no doubt that the streptococci studied by them are identical with *Streptococcus* s.b.e.

Among the fermentation tests employed, two seemed to be of significance and aid in identifying *Streptococcus* s.b.e. The majority (34) of these organisms fermented inulin, whereas a minority (14) fermented raffinose. The ability to ferment inulin but not raffinose is a unique combination of fermentation characteristics among the recognized species of streptococci. Even though appreciable variation was experienced in the fermentation of these substances, they were of great help in recognizing this variety of streptococcus. If a streptococcus from endocarditis was found to ferment inulin but not raffinose, it invariably proved to be *Streptococcus* s.b.e.

STREPTOCOCCUS S.B.E. FROM OTHER SOURCES

Since the great majority of the streptococci from subacute bacterial endocarditis reported in the literature have been found to be of the viridans type, it

has been generally assumed that the primary source of these organisms is the mouth and throat. In line with this assumption, efforts were made to isolate *Streptococcus* s.b.e. from the normal human throat. Swabs were made around the tonsillar areas of 20 healthy individuals. Also, throat swabs from one dog and one cat were included in the experiment. The swabs were rinsed in a nutrient broth and plated immediately by the loop dilution technique on 5 per cent sucrose agar. The plates were incubated at 37 C for 24 hours. In order to avoid *Streptococcus salivarius* only those colonies showing no evidence of marked slime formation were isolated. Of the 680 streptococcus cultures isolated from these plates not a single one was found to be *Streptococcus* s.b.e.

In another attempt advantage was taken of the fact that most cultures of *Streptococcus* s.b.e. are much more tolerant to bile than the ordinary streptococci commonly found in the human throat. Swabs from the throats of ten healthy individuals were lightly streaked over the surface of blood agar containing 30 per cent bile. After incubation for 24 hours the surface growth was washed off with sterile broth and plated immediately by loop dilution on blood agar. Colonies were picked which showed greening reactions. Of the 42 streptococcus cultures isolated, not one was *Streptococcus* s.b.e.

In a third attempt throat swabs from 19 healthy individuals were plated on a blood agar containing 0.02 per cent sodium azide in order to aid in eliminating the growth of most of the organisms in the human throat other than those of the genus *Streptococcus*. After 48 hours' incubation 98 colonies, not of the indifferent type, were isolated. None of these proved to be *Streptococcus* s.b.e. Therefore, if *Streptococcus* s.b.e. exists in the normal human throat, the numbers present must be very small indeed.

Probably quite by accident, a culture of *Streptococcus* s.b.e. was isolated from the irrigations of a chronically infected maxillary sinus. This culture was identified by both physiological and serological methods. Washings from the infected sinuses of eight other individuals, however, yielded only *Streptococcus pyogenes*.

Another culture of *Streptococcus* s.b.e. has been isolated from an extracted tooth. This culture, which was sent to us by Dr. Loewe, was recovered from a patient suffering from subacute bacterial endocarditis caused by *Streptococcus* s.b.e. Other than these two cultures, strains of *Streptococcus* s.b.e. have not been isolated from any other source than the blood stream or heart of endocarditis patients.

NUTRITIVE REQUIREMENTS

Streptococcus s.b.e. grows somewhat more slowly than most streptococci on ordinary laboratory media. Another annoying feature of this organism is that it tends to die out rapidly in liquid media, requiring frequent transfers when broth cultures are used.

This organism, however, does not appear to be very fastidious in its nutritive requirements. Eighteen of the 20 cultures tested produced satisfactory growth upon serial transfer in a casein hydrolyzate medium, plus all the known B

vitamins. Two strains were studied in some detail as to their vitamin requirements. For both strains nicotinic acid, riboflavin, biotin, pyridoxine, and pantothenic acid were found to be essential for growth.

DISCUSSION

As a possible means of preventing the entry of *Streptococcus* s.b.e. into the blood stream with subsequent infection of the heart valves, it would be of great interest to determine the usual habitat of this organism. The results of the present investigation would seem to indicate that this group of streptococci is not a normal inhabitant of the human throat. Of the 820 streptococcus cultures isolated by selective procedures from the human throat not a single member of this group was found. In connection with independent investigations in this laboratory a very large number of viridans streptococci have been studied from several different sources. None of these streptococci has been found to be *Streptococcus* s.b.e. with the exception of the culture from an infected sinus. The *Streptococcus* s.b.e. culture received from Dr. Loewe which was isolated from an extracted tooth of a patient afflicted with endocarditis might be of some significance.

The fact that most strains of *Streptococcus* s.b.e. are relatively tolerant to bile would suggest that these organisms could survive, and possibly grow, in the human intestine. No direct attempts were made to isolate *Streptococcus* s.b.e. from this source.

Lamanna (1944) has studied a streptococcus strain from subacute bacterial endocarditis in some detail as to its morphological variations. An explanation was offered for the diphtheroid appearance of this strain when grown under certain conditions. This culture was received from Dr. Lamanna and found to be a typical strain of *Streptococcus* s.b.e.

Although there exists some degree of physiological variation among the different strains of *Streptococcus* s.b.e., no single culture was encountered which could not be recognized by its physiological pattern alone. In every instance its identity was confirmed by serological methods. Because of its unique display of physiological characteristics, the ease with which it can be characterized serologically, and its apparent medical significance, it would seem fitting to attach to this group of streptococci a specific name. White (1944) has proposed *Streptococcus sanguis*, which would appear to be an appropriate descriptive name. From the biological standpoint the group appears to be as homogeneous and distinct as many of the well-established species in the genus *Streptococcus*. Only by a study of a much larger collection of this group will the validity of a species name be ascertained.

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SUMMARY

A description of a hitherto unrecognized group of streptococci associated with subacute bacterial endocarditis is given. The streptococcus is of the viridans type, having a unique combination of physiological characteristics, including the ability to synthesize large amounts of a polysaccharide in a sucrose broth, to hydrolyze arginine, and to ferment inulin but not raffinose. The species name *Streptococcus sanguis*, suggested by White (1944), seems appropriate.

This streptococcus was recovered from approximately one-third of the cases studied.

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STREPTOCOCCUS S.B.E.: IMMUNOLOGICAL CHARACTERISTICS

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A detailed description of the physiological characteristics of *Streptococcus* s.b.e., a hitherto unrecognized group of streptococci associated with subacute bacterial endocarditis, has been given in a previous publication (White and Niven, 1946). Since these streptococci seemed to comprise a relatively homogeneous group as judged by physiological tests, it seemed desirable to determine their serological properties.

The results of this study have shown that 37 of the 42 cultures studied fall into one serological type, whereas the remaining 5 strains fall into a second type. The interrelationships existing between these two types, as determined by the precipitin and agglutinin techniques, will be discussed.

METHODS

Antisera were prepared by injecting heat-killed cells intravenously into rabbits in accordance with the technique of Lancefield (1933). The cells were harvested from 18-hour meat infusion broth cultures containing 0.1 per cent glucose and 0.2 per cent K_2HPO_4 , resuspended in $\frac{1}{2}$ volume of saline, and killed by heating to 56 C for 1 hour. One to two ml. of this suspension was injected daily for 7 days, followed by a week's rest period. Usually 3 series of injections were sufficient for obtaining satisfactory serum.

In the precipitin experiments the procedure of Lancefield (1933) was followed with respect to the preparation of cell extracts and the technique of testing. When absorption procedures were used, the technique of Lancefield (1938) was employed.

The agglutination experiments were performed by using cellular antigens prepared in the same manner as for animal inoculation. All cell suspensions were adjusted to approximately uniform turbidities before testing.

In studying the immunological characteristics of "*Streptococcus* MG," a streptococcus recovered from cases of primary atypical pneumonia, Mirick *et al.* (1944a) reported that washed cells of these organisms showed a much higher agglutinative titer than unwashed cells. In preliminary experiments no effect was observed in the titer by washing the cellular antigens of *Streptococcus* s.b.e. Therefore, the washing procedure was not followed throughout this study. After mixing the cells with an equal volume of the various serum dilutions, the vials were incubated for 8 to 12 hours at 37 C before the final observations were made.

RESULTS

Preliminary results with a group of 8 cultures of *Streptococcus* s.b.e. indicated a marked degree of serological homogeneity. Extracts of all cultures gave

positive precipitin tests with an antiserum prepared against one strain of this group. However, as more cultures were collected, an occasional strain was found which failed to react with the serum. In all, 5 such cultures have been encountered thus far.

Upon injecting a rabbit with one of the nonreacting cultures, a serum was obtained which reacted with extracts of the remaining 4 cultures. For the sake of convenience we have arbitrarily designated the larger group of the serologically specific *Streptococcus* s.b.e. cultures as type I; the 5 remaining cultures are referred to as type II.

Upon testing the entire collection with the type II serum, we made an interesting observation. As shown in table 1, 5 of the cultures which had shown positive precipitin tests with the type I serum also reacted with the type II serum. Thus it would appear that these 5 cultures contained both types I and II antigens. Accordingly, they have been labeled type I-II, *Streptococcus* s.b.e.

A logical explanation of this occurrence would be that the 5 type I-II cultures were actually impure and composed of a mixture of both types I and II strains.

TABLE 1

Precipitin reactions of Streptococcus s.b.e. extracts with sera prepared against three representative cultures

EXTRACTS TESTED	SERUM USED		
	P5 (type I)	JH49 (type II)	P25 (type I-II)
32	+	—	+
5	—	+	+
5	+	+	+

In order to test this hypothesis, one of these cultures was plated out on an agar medium by the loop dilution technique. Eighteen isolations were made from as many well-separated colonies. Extracts of all these freshly isolated strains reacted positively with both type-specific sera. Therefore, it would seem highly probable that the type I-II cultures possess both type-specific antigens.

From these findings it would appear likely that an antiserum prepared against one of the type I-II cultures would react with the entire collection of *Streptococcus* s.b.e. organisms. As shown in table 1, such a serum has been produced. The serum, however, reacted noticeably stronger with type I extracts than with those prepared from type II cultures.

The presence of both type-specific antigens in one culture was further confirmed by absorption experiments. Both a type I and a type II serum were absorbed with a concentrated cell suspension prepared from a culture representing the so-called type I-II. Table 2 shows that both sera were entirely voided of their respective antibodies, and that they no longer reacted with any of the cultures tested.

Another means of demonstrating the presence of dual antigens in these cul-

tures was accomplished by a second absorption experiment. Two portions of a serum prepared against one of the type I-II cultures were absorbed by type I and type II cells, respectively. The serum absorbed with type I cells no longer reacted with any type I extracts tested, but continued to show positive precipitin

TABLE 2

Absorption experiment showing the removal of precipitin antibodies from types I and II sera with one culture

RABBIT SERUM	CULTURE EXTRACT AND TYPE REPRESENTED	PRECIPITIN REACTION
Type I, unabsorbed	P5(I)	++
	P24(I-II)	++
	JH49(II)	-
Type I, absorbed with P25 (type I-II) cells	P5(I)	-
	P24(I-II)	-
Type II, unabsorbed	P5(I)	-
	P24(I-II)	++
	JH49(II)	++
Type II, absorbed with P25 (type I-II) cells	P24(I-II)	-
	JH49(II)	-

TABLE 3

Absorption experiment demonstrating the presence of two type-specific antibodies in a serum produced against one culture

RABBIT SERUM AGAINST P25 (TYPE I-II)	CULTURE EXTRACT AND TYPE REPRESENTED	PRECIPITIN REACTION
Unabsorbed	P5(I)	++
	JH40(II)	+
	P24(I-II)	++
Absorbed with P5 (type I cells)	P5(I)	-
	JH40(II)	+
	P24(I-II)	+
Absorbed with JH49 (type II) cells	P5(I)	+
	JH40(II)	-
	P24(I-II)	++

tests with the type II extracts. The opposite was true when the serum was absorbed with type II cells (table 3).

The type-specific sera prepared against *Streptococcus* s.b.e. strains appear to be specific for this group of organisms when tested by the Lancefield precipitin technique. A number of streptococci representing the various Lancefield groups, A through G, have been tested with negative results. The entire col-

lection of streptococci from subacute bacterial endocarditis has been tested, with no single culture showing cross reactions; the only reactions obtained were with those streptococci which had been previously identified as *Streptococcus* s.b.e. by virtue of their physiological characteristics. Also included in the survey were various members of the well-defined species of the viridans group of streptococci, including *Streptococcus salivarius* and *Streptococcus mitis* from the human throat. Again no cross reactions were obtained.

Because of the likelihood that the agglutination technique may be more convenient to perform than the precipitin test in some laboratories, the possibility of identifying *Streptococcus* s.b.e. by this method was determined. The results of this study have revealed the presence of a multiplicity of agglutinative

TABLE 4

Absorption experiments demonstrating complexity of agglutinative antibodies in a type I Streptococcus s.b.e. serum

RABBIT SERUM	CELL SUSPENSION AND TYPE REPRESENTED	SERUM DILUTION								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
Type I, unabsorbed	P5(I)	3*	4	4	4	4	4	4	4	2
	JH49(II)	3	4	4	4	3	1	0	0	0
	P25(I-II)	4	4	4	3	2	1	0	0	0
Type I, absorbed with JH49 (type II) cells	P5(I)	4	4	4	4	4	4	4	4	4
	JH49(II)	0	0	0	0	0	0	0	0	0
	P25(I-II)	4	4	4	4	3	2	0	0	0
Type I,† absorbed with P24 (type I-II) cells	P5(I)	4	4	4	4	3	3	1	0	0
	JH49(II)	0	0	0	0	0	0	0	0	0
	P25(I-II)	0	0	0	0	0	0	0	0	0

* 0 to 4 indicates degree of agglutination.

† A different lot of type I serum with lower titer was used for this absorption.

antigens among these streptococci, a condition which reminds one of similar situations existing in certain groups of the *Enterobacteriaceae*.

Rabbit sera with relatively high titers of agglutinins for homologous cellular antigens could be obtained with 2 or 3 series of injections. For example, the type I serum used throughout most of this study agglutinated all type I cells tested in a serum dilution as high as 1:10,240. In addition, this serum also agglutinated both types II and I-II cells, but at a much lower dilution (1:640). Since types II and I-II cells were agglutinated at approximately the same titer, it might be assumed that they contained similar, or identical, antigens. That this is not the case is shown in table 4. The type I serum absorbed with type II cells continued to agglutinate type I *Streptococcus* s.b.e. and also all members in type I-II. On the other hand, a type I serum absorbed with type I-II cells would no longer agglutinate type II cells. Unfortunately for the latter absorption experiment, another lot of type I serum had to be used because of the depletion of the original lot. Consequently, the results tabulated in table 4 make

it appear that absorption with type I-II cells reduced the titer of the serum for type I cells also. This was not the case.

Table 5 demonstrates a similar situation when a type II serum was absorbed with type I and type I-II cells, respectively. In harmony with reports from

TABLE 5
Absorption experiments showing complexity of agglutinative antibodies in a type II Streptococcus s.b.e. serum

RABBIT SERUM	CELL SUSPENSION AND TYPE REPRESENTED	SERUM DILUTION						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Type II, unabsorbed	JH52(II)	4*	4	4	4	2	1	0
	JH58(I)	4	4	3	0	0	0	0
	P25(I-II)	4	4	3	2	2	1	0
Type II, absorbed with P5 (type I) cells	JH49(II)	4	4	4	4	3	3	3
	JH44(I)	1	0	0	0	0	0	0
	P24(I-II)	3	2	2	1	0	0	0
Type II, absorbed with P25 (type I-II) cells	JH49(II)	4	4	4	3	3	2	1
	P5(I)	1	0	0	0	0	0	0
	JH19(I-II)	0	0	0	0	0	0	0

* 0 to 4 indicates degree of agglutination.

TABLE 6
Absorption experiments demonstrating complexity of agglutinative antibodies in a type I-II Streptococcus s.b.e. serum

RABBIT SERUM	CELL SUSPENSION AND TYPE REPRESENTED	SERUM DILUTION							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Type I-II, unabsorbed	P25(I-II)	4*	4	4	4	4	3	2	1
	P5(I)	3	2	2	2	2	1	0	0
	JH40(II)	4	4	4	4	3	2	1	1
Type I-II, absorbed with P5 (type I) cells	P24(I-II)	3	3	3	3	3	3	2	1
	JH44(I)	0	0	0	0	0	0	0	0
	JH40(II)	3	3	3	2	2	2	1	0
Type I-II, absorbed with JH49 (type II) cells	P25(I-II)	4	4	4	4	4	2	0	0
	P5(I)	2	2	2	1	0	0	0	0
	JH40(II)	1	0	0	0	0	0	0	0

* 0 to 4 indicates degree of agglutination.

other laboratories, we have observed that absorption of a serum with a heterologous type may actually increase its titer for organisms of the homologous type.

Another series of agglutination experiments was performed on the serum against a type I-II *Streptococcus s.b.e.* As shown in table 6, the titer of the serum for type II cells was as high as it was with the homologous culture. Type I cells agglutinated in somewhat lower serum dilutions. Absorption with either type

I or type II cells removed the agglutinative antibodies for the respective type only.

Although the antigenic complex of the different serological types of *Streptococcus* s.b.e. appears to be somewhat involved, the picture may be visualized in the following manner. Both types (as well as type I-II) appear to have a minimum of three agglutinative antigens. The three agglutinative antigens of type I *Streptococcus* s.b.e. might be labeled A, B, and C; type II would have antigens A, D, and E; and type I-II, antigens A, C, and D. All *Streptococcus* s.b.e. cultures, then, have one common agglutinative antigen, whereas those cultures in type I-II would have two agglutinative antigens common to either type I or type II.

A number of other streptococci from subacute bacterial endocarditis have been tested for their ability to cross-agglutinate with the various *Streptococcus* s.b.e. type sera. Although the majority showed no evidence of agglutination, an occasional culture of *Streptococcus mitis* was found which agglutinated with the sera in relatively high dilutions.

Mirick *et al.* (1944b) reported that type I *Streptococcus salivarius* cultures cross-reacted with their sera prepared against *Streptococcus* MG, as demonstrated by both the agglutination and precipitin techniques. It is interesting to note that *Streptococcus salivarius* also cross-agglutinates with the *Streptococcus* s.b.e. sera in relatively high dilutions. *Streptococcus salivarius* cells representing three serological (precipitin) types cross-agglutinated with all three *Streptococcus* s.b.e. sera used in this study, positive results sometimes occurring in serum dilutions as high as 1:320. Conversely, types I and II *Streptococcus* s.b.e. cultures cross-agglutinated with types I and II *Streptococcus salivarius* sera. It should be recalled, however, that *Streptococcus salivarius* does not show a positive precipitin test with the *Streptococcus* s.b.e. sera.

The cross reactions with *Streptococcus salivarius* should by no means imply that *Streptococcus* MG and *Streptococcus* s.b.e. are related organisms. They are known to be two serologically and physiologically distinct groups of streptococci.

These cross agglutinations with *Streptococcus salivarius* and occasional strains of *Streptococcus mitis* would tend to detract from the usefulness of the agglutination technique as a means of identifying *Streptococcus* s.b.e. As reported previously (Niven and White, 1946), *Streptococcus mitis* was recovered from approximately the same number of cases of endocarditis as was *Streptococcus* s.b.e. No *Streptococcus salivarius* cultures were encountered, however.

DISCUSSION

In harmony with many other studies upon the viridans streptococci in this and other laboratories, *Streptococcus* s.b.e. appears to have no group-specific antigen that would correspond to the "C" substance found in the hemolytic and other groups of streptococci. From the results with the type I-II streptococcus it might appear that this organism possesses a group-specific antigen, since a serum prepared against one of these cultures shows positive precipitin tests with all *Streptococcus* s.b.e. extracts. However, the absorption experiments would seem to indicate the presence of two type-specific antigens rather than a group antigen.

Since two serological types among the 42 cultures of *Streptococcus* s.b.e. have been found, it would be natural to expect that more types exist and will be found as more cultures are studied. In spite of this probability, serological methods should prove to be useful in the study of streptococci recovered from endocarditis. A serum prepared against a type I-II strain might be of value from the practical standpoint.

There seems to be no tendency for individual strains of *Streptococcus* s.b.e. to lose the type-specific antigens. Some of the cultures have been transferred at occasional intervals in the laboratory for over two years with no evidence in loss or change of type antigen. One type I strain was serially cultured 40 times in a broth containing 50 per cent homologous serum with no success in inducing loss of the type-specific antigen.

SUMMARY

The 42 cultures of *Streptococcus* s.b.e. recovered from cases of subacute bacterial endocarditis fall into two serological types as determined by the precipitin technique.

Five cultures were found which gave positive precipitin tests with both type I and type II antisera. A serum prepared against one of these strains reacted with all 42 cultures. This appears to be due to the presence of antigens of both types in the cells of these strains, rather than to the presence of a group-specific antigen.

There appears to be a number of agglutinating antigens in the individual strains of *Streptococcus* s.b.e. The agglutination test, however, is not suitable for the identification of this organism.

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PRODUCTION OF CONIDIA IN SUBMERGED CULTURES OF *PENICILLIUM NOTATUM*¹

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Foster, McDaniel, Woodruff, and Stokes (1945) recently described the formation of conidia by molds of the genus *Penicillium* during submerged growth in liquid culture media. Controlling or associated factors for this phenomenon were described as the presence of a rather high calcium-ion concentration and, in general, the development of considerably less mycelium before spore formation than is found if conditions favor development of the vegetative phase alone.

In the course of experimental studies on microbiological problems relating to the production of penicillin, we have observed that submerged conidia formation may be induced in nutrient media of normal calcium concentrations. Media containing nitrogen supplied by ground whole wheat, malted wheat, stillage from yeast-fermented wheat mash, or corn steep water have all resulted in formation of conidia by the submerged mycelium under proper conditions.

A typical liquid substrate in which submerged sporulation by *Penicillium notatum* has been reproduced consists of 25 per cent thin-grain stillage² and 3 per cent glucose, with the initial pH adjusted to 5.5 with NaOH. When incubated at 24 C and shaken to produce submerged growth, conidia formation was evident in cultures within 48 hours and was heaviest during the fourth and fifth days. The presence of 0.025 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 per cent KH_2PO_4 , or 0.004 per cent $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ did not noticeably affect the production of conidia, whereas the addition of 0.3 per cent NaNO_3 resulted in much heavier mycelial growth and only very slight sporulation. Penicillin titers were generally low (20 units per ml or less) in sporulating submerged cultures.

Factors favoring conidia formation in submerged cultures on media such as that described above appear to include a moderate amount of growth and the maintenance of an hydrogen-ion concentration in the culture medium of about pH 5.0 to pH 6.5. Only a sparse formation of conidia occurred when vegetative development was sufficiently heavy to interfere with good shaking and, therefore, with good aeration. Extensive sporulation occurred, however, in cultures of slight or moderate growth. The relationship between extent of growth and sporulation is in general agreement with the observations of Foster *et al.* (1945). The acid medium is usually associated with the presence of readily utilizable carbohydrates, such as glucose, and the accompanying formation of organic acids.

¹ *Penicillium notatum* NRRL 832.

² Thin-grain stillage from yeast-fermented wheat mash having the proximate composition: 4.2 per cent total solids, 47.0 per cent protein (d.b.), 2.3 per cent sugar as glucose (d.b.).

Conidia formation in submerged culture has also been observed in a corn steep water lactose medium which contained added iron to the extent of about 500 μg per ml or more. Table 1 shows the effect of iron on submerged conidia and penicillin formation employing shaken flasks incubated at 24 C.

Foster *et al.* (1945) showed that 0.5 to 5.0 per cent calcium chloride, or 0.18 to 1.8 per cent calcium, was very favorable in stimulating the production of conidia by submerged culture in two species of *Penicillium*. They also mentioned that "heavy metal nutrition of the organism also is influential to some extent." Foster (1939), in reviewing the heavy metal nutrition of fungi (surface growth), indicated that iron was generally essential for the fructification of molds but that zinc sometimes suppressed it. Our work showed that 0.05 to 0.1 per cent iron resulted in submerged conidia formation, whereas 0.2 per cent iron suppressed

TABLE 1

The effect of added iron on conidia formation, pH, and penicillin production by Penicillium notatum NRRL 832

IRON ADDED*	AGE IN DAYS						CONIDIA FORMATION	VEGETATIVE GROWTH
	4		5		6			
	pH	units/ml	pH	units/ml	pH	units/ml		
<i>μg/ml</i>								
0	7.5	42	7.2	51	7.2	52	—	+
100	7.3	34	7.1	54	7.1	54	—	+
500	6.8	12	6.6	18	6.5	20	+	+
750	6.5	11	5.8	14	5.5	9	+	+
1,000	5.8	6	4.8	4	4.7	2	+	+
2,000	3.0	0	3.3	0	3.1	0	—	—

* The basal medium consisted of 4 g of corn steep water and 2 g of lactose per 100 ml, pH 5.5; 180 ml per 1,000-ml flask. The iron content of the basal medium was about 20 μg per ml or about 0.002 per cent. The calcium content was about 40 μg per ml. Iron was added as ferric alum with the exception of the 2,000 μg per ml flask in which the iron was added as ferric nitrate.

growth almost completely. Thus, on a weight basis, iron is about four times as effective in promoting submerged sporulation as is calcium at minimal levels.

SUMMARY

The formation of conidia in submerged shake-flask cultures of *Penicillium notatum* has been induced in a variety of media, without the addition of heavy metals, under conditions favoring slight or moderate growth, thus permitting good aeration, and the maintenance of an hydrogen-ion concentration between pH 5.0 and pH 6.5. Submerged sporulation was brought about under these conditions in media supplied with nitrogen by corn steep water, ground wheat, malted wheat, or thin-grain stillage.

The addition of iron to a basal corn steep water lactose medium to the extent of about 100 μg per ml did not materially interfere with penicillin production or

promote conidia formation, but additions to the extent of 500 to 1,000 μg per ml suppressed penicillin titers, lowered pH, and promoted submerged sporulation. The addition of 2,000 μg per ml strongly suppressed the growth of the mold, and no conidia were produced under the conditions of the experiment.

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THE DISTRIBUTION OF ENTERIC STREPTOCOCCI

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Comparatively little work has been done to determine the frequency distribution of enteric streptococci in nature. It is generally considered that the enterococci have their origin in the intestinal tract of man and other warm-blooded animals. Horses and cattle have been mentioned by several investigators as sources of these organisms, but the occurrence of fecal streptococci in most warm-blooded animals remains a matter of conjecture. Although enterococci of the *Streptococcus faecalis* and *Streptococcus liquefaciens* types have been shown to occur on plants (Sherman, 1937a), their presence on vegetable life and in the soil does not necessarily indicate that these are their natural habitats. The presence of enteric streptococci on plants and in the soil is more apt to be an indication of survival than of growth and reproduction. A knowledge of the frequency distribution of fecal streptococci in animals, together with comparatively simple laboratory methods of isolation and identification, would help to determine the public health and sanitary significance of this group of microorganisms. The finding of fecal streptococci in adequately treated swimming pool water, and in the absence of demonstrable coliform organisms, suggests that they either are more resistant to the chemical treatment (Hajna and Perry, 1943) or occur in greater numbers than is suspected. In either case the enterococci take on an added sanitary significance.

EXPERIMENTAL

The work of Sherman (1937a), Snyder and Lichstein (1940), and Hajna and Perry (1943) suggested bacteriological media and laboratory methods of analysis for the demonstration of enterococci. As a consequence of their findings the "SF" medium of Hajna and Perry, employing 0.05 per cent sodium azide as an inhibitor of gram-negative microorganisms, and an incubation temperature of 45.5 C (113.9 F) was used in the initial experiments. The demonstration by Sherman (1937a) that enterococci are capable of growth and reproduction in the presence of 6.5 per cent sodium chloride led to its use in later experiments. Fifty-three specimens of human and animal feces and soil were examined for fecal streptococci and coliform bacteria. Thirty specimens were examined for fecal streptococci using "SF" medium alone. The remaining 23 specimens were examined on a comparative basis using "SF" medium and "SF" medium containing 6.5 per cent sodium chloride. The source and number of specimens are shown in table 1.

Fresh specimens were weighed into 125-ml, wide-mouth, ground-glass-stoppered bottles, and sufficient sterile physiological salt solution was added to make an initial dilution of 1:10. Subsequent decimal dilutions of from 1:100 to 1:1,000,000 were prepared for seedings. Parallel inoculations were made of the

well-shaken suspensions into "SF" medium, standard lactose broth, and, in the case of 23 specimens, into "SF" medium containing 6.5 per cent sodium chloride. The inoculated tubes of "SF" and modified "SF" media were incubated in a constant temperature water bath at 45.5 C (113.9 F) for 72 hours. One hundred and sixty-one random temperature recordings, made over a period of 22 days, revealed a minimum temperature of 45.0 C (113.0 F) and a maximum temperature of 45.9 C (114.6 F), with an average temperature of 45.45 C (113.8 F). All sodium azide cultures were examined daily, macroscopically for acid production and microscopically for bacterial growth. At the end of the incubation period tubes containing fecal streptococci, with or without acid production, were

TABLE 1
Source and number of specimens

SOURCE	NUMBER OF SPECIMENS	SOURCE	NUMBER OF SPECIMENS
Human.....	5	Rabbit.....	5
Cat.....	5	Rat.....	5
Mouse.....	5	Chicken.....	6
Guinea pig.....	5	Flies.....	3
Dog.....	6	Monkey.....	6
		Soil.....	2

TABLE 2
Selective action of "SF" medium for fecal streptococci

ORGANISMS PRESENT	ACIDITY SODIUM AZIDE BROTH	
	Positive	Negative
Fecal streptococci only.....	15 (9.9%)	6 (3.9%)
Fecal streptococci and rods.....	45 (29.8%)	1 (0.7%)
Rods only.....	11 (7.2%)	
None.....		72 (47.6%)
Totals.....	71	79

streaked on sodium azide agar ("SF" medium containing 1.5 per cent agar). These culture plates were then incubated at 37.5 C (99.5 F) for 24 hours and for 48 hours, and colonies showing acid production were fished for taxonomic study.

The standard lactose broth tubes were incubated at 37.5 C (99.5 F) for 24 and for 48 hours. Tubes in which gas was present at the end of the incubation period were streaked on Levine's eosin methylene blue agar. Following incubation of these culture plates well-isolated coliform colonies were fished for biochemical identification.

RESULTS

A review of the data of the first 30 specimens examined by the "SF" medium revealed that fecal streptococci were present in 26 of the 28 fecal samples. One specimen of mouse feces and one of dog feces failed to show any evidence of

enterococci. The two soil samples failed to produce any evidence of acid production as shown by the bromcresol purple indicator in the medium. Microscopically the tubes from these specimens were negative for any streptococci.

Of the 140 tubes of "SF" medium inoculated from the 28 fecal suspensions, 71 produced evidence of fecal streptococci as indicated by acid production. Fifteen of these tubes (9.9 per cent) contained only fecal streptococci, whereas 45 (29.8 per cent) contained fecal streptococci in combination with rods. Eleven

TABLE 3a

Comparative selective action of "SF" medium and "SF" medium containing 6.5 per cent sodium chloride

ACIDITY BROTH TUBES	MICROSCOPIC GROWTH	MEDIUM							
		"SF" plus 6.5% salt				"SF"			
		Hours				Hours			
		24	48	72	Total	24	48	72	Total
Negative	Negative			45	45			33	33
Negative	Rods only			9	9			4	4
Negative	Fecal strep. only			23	23			18	18
Negative	Fecal strep. and rods			6	6			21	21
Positive	Rods only			1	1	4	1	1	6
Positive	Fecal strep. only	1	16	3	20	2	11	2	15
Positive	Fecal strep. and rods	10	21	3	34	21	18	2	41
Totals		11	37	90	138	27	30	81	138

TABLE 3b

Comparative selective recovery of microorganisms by "SF" medium and "SF" medium containing 6.5 per cent sodium chloride

ORGANISMS PRESENT	ACID REACTION		NO ACID REACTION	
	"SF" plus 6.5% salt	"SF"	"SF" plus 6.5% salt	"SF"
Fecal strep. only	14.5%	10.8%	16.6%	13.0%
Fecal strep. and rods	24.6%	29.7%	4.3%	15.2%
Rods only	0.7%	4.3%	6.5%	2.9%

tubes (7.2 per cent) were false positive, since microscopic examination failed to reveal the presence of streptococci and showed them to contain only rods. In addition to this discrepancy in results 7 tubes (4.6 per cent) in which no acid was produced were found to contain fecal streptococci alone or in combination with rods (table 2).

It thus appeared that bromcresol purple sodium azide broth ("SF" medium) failed as a presumptive medium to reveal the presence of fecal streptococci in approximately 5 per cent and gave a false positive reaction in approximately 7 per cent of the inoculated tubes. It is also of interest that of the 6 tubes which were negative with sodium azide broth (no acid) but positive for fecal streptococci, 4 occurred in the 1:100,000 dilution, 1 in the 1:10,000 dilution, and 1 in

the 1:10 dilution. These results are of numerical significance as will be shown later.

The addition of 6.5 per cent sodium chloride to the "SF" medium had the effect of reducing the number of false positive and negative reactions without any appreciable reduction in the recovery of acid-producing enterococci. Of the 23 specimens examined, both media being used, 21 contained fecal streptococci. One mouse and one rat specimen were negative for enterococci. Of

TABLE 4

Highest dilutions with a positive acid reaction containing either fecal streptococci alone or in combination with rods

SAMPLE NUMBER	"SF" PLUS 6.5% SALT		"SF"	
	Fecal strep. alone	Fecal strep. and rods	Fecal strep. alone	Fecal strep. and rods
31	10^{-4}		10^{-3}	
32	10^{-3}		10^{-3}	
33	Negative	Negative		10^{-1}
34		10^{-1}		10^{-1}
35	10^{-6}			10^{-2}
36	10^{-3}		10^{-3}	
37		10^{-1}		10^{-2}
38	10^{-4}			10^{-4}
39	Negative	Negative	Negative	Negative
40	Negative	Negative	10^{-4}	
41	10^{-5}			10^{-2}
42		10^{-1}		10^{-2}
43	10^{-6}		10^{-3}	
44		10^{-2}	10^{-5}	
45	Negative	Negative	Negative	Negative
46		10^{-1}		10^{-2}
47	10^{-2}			10^{-2}
48		10^{-3}		10^{-2}
49	10^{-3}		10^{-3}	
50	10^{-2}			10^{-3}
51		10^{-2}	10^{-3}	
52	10^{-4}			10^{-3}
53		10^{-2}	10^{-3}	

the 138 tubes of each medium inoculated, 62 tubes (44.8 per cent) of "SF" and 55 tubes (39.8 per cent) of modified "SF" showed acid production. Among the positive cultures in both media, 3.7 per cent more tubes of the modified "SF" than of the original medium contained fecal streptococci without any interfering bacilli. Of greater significance is the marked reduction in the number of cultures giving a false positive reaction from 4.3 per cent to 0.7 per cent.

Both media gave a high percentage of tubes containing enterococci alone or in combination with rods without any macroscopic evidence of acid production. Thirty-nine "SF" tubes (28.2 per cent) and 29 modified "SF" tubes (20.9 per cent) failed to show any evidence of acidity yet contained either pure cultures of enterococci or mixtures of enterococci and bacilli at the end of the 72-hour incubation period (table 3a and b).

A comparison of the two media on the basis of the presumptive (acid) reaction reveals that in the modified "SF" broth 11 specimens (47.8 per cent) contained pure cultures of fecal streptococci in dilutions ranging from 1:100 to 1:1,000,000. In the "SF" medium 9 specimens (39.1 per cent) contained these organisms in

TABLE 5
Comparative incidence of enterococci and Escherichia coli

SOURCE	SAMPLE NUMBER	HIGHEST DILUTION SHOWING		SOURCE	SAMPLE NUMBER	HIGHEST DILUTION SHOWING	
		Enterocci	<i>E. coli</i>			Enterococci	<i>E. coli</i>
Human	1	10 ⁻³	10 ⁻⁵	Rabbit	11	Negative	10 ⁻³
	12	10 ⁻³	10 ⁻⁵		19	10 ⁻¹	10 ⁻³
	51	10 ⁻³	10 ⁻⁴		26	10 ⁻¹	10 ⁻⁵
	52	10 ⁻⁴	Negative		33	10 ⁻¹	Negative
	53	10 ⁻³	10 ⁻⁴		46	10 ⁻²	10 ⁻⁵
Cat	2	10 ⁻³	10 ⁻³	Rat	14	10 ⁻²	10 ⁻⁵
	8	10 ⁻³	10 ⁻⁵		22	10 ⁻¹	10 ⁻⁵
	18	10 ⁻³	10 ⁻⁵		23	10 ⁻¹	10 ⁻⁵
	31	10 ⁻⁵	10 ⁻⁷		37	10 ⁻²	10 ⁻⁵
	40	10 ⁻⁴	10 ⁻⁷		39	10 ⁻²	10 ⁻³
Mouse	3	10 ⁻¹	10 ⁻⁵	Chicken	17	10 ⁻⁴	10 ⁻⁵
	10	10 ⁻³	10 ⁻⁵		24	10 ⁻⁵	10 ⁻⁵
	15	10 ⁻¹	10 ⁻⁵		25	10 ⁻⁵	10 ⁻⁵
	38	10 ⁻⁴	10 ⁻⁵		30	10 ⁻⁵	10 ⁻³
	45	10 ⁻²	10 ⁻³		32	10 ⁻³	10 ⁻³
Guinea pig	4	10 ⁻¹	10 ⁻⁵	Flies	44	10 ⁻⁵	10 ⁻⁵
	9	10 ⁻²	10 ⁻⁵		20	10 ⁻⁴	10 ⁻⁴
	13	10 ⁻²	10 ⁻³		27	10 ⁻⁵	10 ⁻⁴
	34	10 ⁻¹	10 ⁻⁴		28	10 ⁻²	Negative
	42	10 ⁻²	10 ⁻²				
Dog	7	10 ⁻³	10 ⁻⁵	Monkey	36	10 ⁻³	10 ⁻⁵
	16	Negative	10 ⁻⁵		41	10 ⁻⁵	10 ⁻⁵
	21	10 ⁻²	10 ⁻²		47	10 ⁻²	Negative
	29	10 ⁻³	10 ⁻³		48	10 ⁻³	Negative
	35	10 ⁻⁵	10 ⁻⁵		49	10 ⁻³	10 ⁻²
	43	10 ⁻²	10 ⁻³		50	10 ⁻³	10 ⁻⁴
Soil	5	Negative	Negative				
	6	Negative	Negative				

pure culture and in dilutions of from 1:100 to 1:10,000. Fecal streptococci associated with bacilli were found in 8 specimens (52.2 per cent) cultured in modified "SF" broth as compared with 12 specimens (60.9 per cent) in the unmodified medium (table 4).

Forty-six (90 per cent) of the 51 fecal specimens examined for coliform organisms contained *Escherichia coli* in dilutions ranging from 1:100 to

1:10,000,000. Five specimens which did not contain *E. coli* did contain other coliform types. Only 2 of the 51 specimens were negative for enterococci, the remaining 49 samples contained these organisms in dilutions of from 1:10 to 1:1,000,000 (table 5). Among the specimens containing both *E. coli* and enterococci, 8 (15.7 per cent) contained enterococci in greater numbers than *E. coli*, 11 (21.6 per cent) contained these organisms in equal numbers, and 32 (62.7 per cent) contained greater numbers of *E. coli* than of fecal streptococci (table 6).

DISCUSSION

Although it has been suspected that the enterococci are rather generally distributed in the intestinal tract of warm-blooded animals, earlier work has been confined to a very few animal species. This study demonstrates that fecal streptococci are common in the excreta of 10 animal species and that they occur in

TABLE 6
Numerical occurrence of Escherichia coli and enterococci

SPECIMENS IN WHICH	NUMBER OF SAMPLES
<i>E. coli</i> less than enterococci.....	8 (15.7%)
<i>E. coli</i> equal enterococci.....	11 (21.6%)
<i>E. coli</i> greater than enterococci by 1 dilution.....	7 (13.8%)
<i>E. coli</i> greater than enterococci by 2 dilutions.....	11 (21.6%)
<i>E. coli</i> greater than enterococci by 3 dilutions.....	5 (9.8%)
<i>E. coli</i> greater than enterococci by 4 dilutions.....	8 (15.6%)
<i>E. coli</i> greater than enterococci by 5 dilutions.....	1 (1.9%)
Total.....	51

significant numbers. Though generally outnumbered by *E. coli*, the resistance of enterococci to chemical agents and possibly to other environmental factors make them of sanitary significance as indices of fecal contamination and pollution.

"SF" medium is of great value in the detection of fecal streptococci in natural sources, but it is deficient in that false positive (acid) reactions occur and in that fecal streptococci may be present in the absence of a positive (acid) reaction. Modification of the "SF" medium by the addition of 6.5 per cent sodium chloride results in the elimination of most false positives but does not appreciably influence the occurrence of false negative reactions. Studies should be undertaken looking toward the development of a modified medium with which greater recovery of enterococci can be obtained without any sacrifice in specificity.

SUMMARY

Fifty-one fecal specimens and two soil samples were examined for enterococci and coliform organisms. The two soil samples were negative for both enterococci and *Escherichia coli*. Forty-nine specimens representing 10 animals contained enterococci in from one-tenth to one one-millionth of a gram of feces. *E. coli*

was present in 46 specimens and was recovered in dilutions ranging from 1:100 to 1:10,000,000.

Acidity was produced in 71 tubes of "SF" broth, of which 60 contained fecal streptococci alone or in combination with rods. Eleven tubes were false positive (acid), containing bacilli but no streptococci. Four and six-tenths per cent of the inoculated tubes were false negative (no acid), containing fecal streptococci without evidence of acid production.

The addition of 6.5 per cent sodium chloride to the "SF" medium reduced the number of false positive reactions from 4.3 per cent to 0.7 per cent, but did not appreciably influence the occurrence of false negative reactions.

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A NEW TELLURITE PLATING MEDIUM AND SOME COMMENTS ON THE LABORATORY "DIAGNOSIS" OF DIPHTHERIA

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It is evident that the traditional laboratory examination for the diphtheria bacillus, based on the microscopic appearance of a stained smear from a culture on Loeffler's medium, is in every respect as subjective a matter as is the clinical diagnosis of the case by the physician. In each instance the decision is based largely on a visual image interpreted in the light of past experience with such images. It is true that the clinician has additional supporting evidence, involving touch, hearing, and even smell to assist him. The bacteriologist depends on sight alone, and his report will be correct in proportion to his earlier experiences in similar interpretations. Diphtheria, however, has become such a rare condition in some areas that a generation of bacteriologists whose encounters with the organism have been relatively infrequent are replacing those of twenty years ago whose mornings were usually devoted to that particular matter.

Moreover, it usually happens that the sudden appearance of a few cases of diphtheria in an area from which the disease has been absent for some time will result in a flood of throat cultures from contacts and possible carriers, as well as from all types of nondiphtheritic pharyngitis. The majority of these will be negative, but in each case a time-consuming search of a smear must be made before it can be so pronounced.

These facts have resulted in the gradual substitution, in many laboratories, of a procedure which is much more rapid and somewhat simpler in subjective interpretation than the use of Loeffler's cultures. This method depends on the inhibition by potassium tellurite of most throat organisms and on the rather characteristic colonial appearance of members of the *Corynebacterium* group, which grow well in its presence. An imposing array of formulae for tellurite media has already grown up in the literature of the past fifteen years. The writers hesitate somewhat to add one more to the list but are persuaded to do so because of the conviction that to be reasonably practicable a diagnostic medium should be relatively easy to prepare and reproducible in its results. The two portions of the medium to be described are commercially available, one in dehydrated form, the other as a fluid concentrate,² and its preparation from this source is extremely simple. In the absence of a commercial source, the medium may still be pre-

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² Difco Laboratories, Inc., Detroit, Mich.

pared in the average laboratory, although it is suggested that the matter be entrusted to, or at any rate supervised by, a chemically trained worker.

The formula is based upon the known growth requirements of members of the diphtheria bacillus group (Mueller, 1940) and is still makeshift in certain respects because of practical considerations and incomplete knowledge of certain nutritional requirements (Cohen, Snyder, and Mueller, 1941). None of the ingredients is sharply critical from the quantitative aspect. Even the potassium tellurite permits reasonable departure from the specified amount, and comment on this point will be found in a later section.

Composition of the medium

Agar base	
	Quantities for 500 ml
Agar.....	10 g
Casamino acids, Technical ³	10 g
Commercial casein ⁴	2.5 g
KH ₂ PO ₄	0.35 g
MgSO ₄ ·7H ₂ O.....	0.05 g
Tryptophane.....	0.025 g
Water to 500 ml	
Serum-tellurite	
	Stock solution sufficient for 4 liters of medium
Sodium lactate solution ⁵	40 ml
Ethyl alcohol.....	10 ml
Calcium pantothenate.....	0.2 mg
Sterile horse or beef serum.....	50 ml
Potassium tellurite.....	0.4 g

In order to prepare the medium from its component parts, a stock solution of the "serum-tellurite" should first be made. Proceed as follows: Place sodium lactate in a suitable container and sterilize in the autoclave. After cooling, add, with sterile precautions, (1) the alcohol, (2) calcium pantothenate sterilized by autoclaving in a few ml of water, (3) the serum, either prepared with sterile precautions or passed through a Berkefeld or Seitz filter, and (4) the potassium tellurite dissolved in a few ml of sterile water. It appears to be of no consequence whether the tellurite solution is entirely clear. Mix thoroughly after each addi-

³ Trade name for a hydrochloric acid hydrolyzate of casein supplied by Difco Laboratories, Inc., Detroit, Mich. The preparation of a comparable material is described by Mueller (1939).

⁴ Suspend 5 g dry commercial casein in 50 ml water, add strong (40 to 50 per cent) NaOH drop by drop slowly, with shaking, until casein is in complete solution. It requires 10 to 15 minutes to bring this about, and an excess of NaOH should be avoided.

⁵ The sodium lactate solution here specified is prepared in the following way. (There is no reason to suppose that a commercial sodium lactate could not be substituted by making the necessary readjustment of quantity.) Fifty ml of 85 per cent lactic acid plus 75 ml H₂O with a few drops of phenol red solution are neutralized with strong (40 to 50 per cent) NaOH. The solution is heated just to boiling for 5 minutes, adding more NaOH if necessary to retain red color (decomposition of lactone?). Forty ml of the resulting solution is used.

tion, stopper well, and store in the cold room. The slow formation of some insoluble material does the solution no harm, and it seems to make no difference whether it is shaken up or not before use. Excessive heating is to be avoided with this as with other media made up from an amino acid base.

The agar base should, therefore, be freshly prepared for each batch of plates. The ingredients listed above, in the stated quantities, are mixed in a flask with sufficient water to make 500 ml. The pH is adjusted to about 7.6. If the dehydrated base is available, 22.5 grams are simply added to 500 ml of water. In either case the flask is heated to boiling with frequent shaking, transferred while still hot to the autoclave, and sterilized at 10 pounds' steam pressure for 10 minutes. Cool the freshly sterilized solution to about 50 C and add 12.5 ml of the serum-tellurite solution. Mix, pour reasonably thick plates (20 ml), and allow to cool without covers for a few minutes. The surface of the plates must be dry.

The foregoing medium is the outgrowth of four years of practical use of tellurite plates in the detection of diphtheria carriers. Under a project conducted in this laboratory at the request of the Epidemiological Board of the Preventive Medicine Division of the Surgeon General's Office, United States Army, approximately 400 cultures a week during this period have been taken at military posts and examined for pathogenic respiratory bacteria, including *Corynebacterium diphtheriae*. The extremely low rate of carriage of virulent diphtheria organisms (about 1 in 1,000 cultures) led us to examine rather critically the method in use. This consisted originally in the use of another type of tellurite plate, which, however, showed considerable variability from one lot to another in colonial appearance, particularly of "gravis" type of *C. diphtheriae*. To escape this difficulty, and to devise a formula capable of exact duplication at any time and place, regardless of the availability of any specific commercial product, a peptone-free medium containing only materials commonly available to most laboratories seemed desirable. It was sought, moreover, so to constitute the medium that the characteristic differences between mitis, gravis, and intermedius types of *C. diphtheriae* would be accentuated. To a degree these objectives are believed to have been accomplished.

The medium contains at least two factors necessary for growth, particularly of the gravis type, from minute inocula. One of these is contained in the whole commercial casein, the other in the horse or beef serum. Should it eventually be possible to supply these components in a pure form or as concentrates, the preparation may be simplified and the medium probably improved, since for practical reasons the amounts of both substances now recommended are suboptimal. The addition of pantothenic acid may be unnecessary, as sufficient is probably supplied by these two ingredients. Since an excess does no harm, it has seemed wise to include it in the formula because it is essential to the growth of many strains.

The proportions of sodium lactate and alcohol have been empirically balanced so that an adequate energy source is available in such form that extreme alterations in pH do not occur. Utilization of lactate tends to cause a marked rise in

pH (Na_2CO_3), whereas with glucose or alcohol the trend is more or less strongly toward an acid reaction. The selection of ethyl alcohol was influenced in part by the belief that in the amount present in the fluid concentrate it would supplement the tellurite in suppressing growth of accidental contaminants.

The concentration of potassium tellurite deserves particular comment. In certain of the published formulae great emphasis is laid on the necessity for an accurate assay of each lot of this salt, and a careful adjustment of the quantity to be used. Different batches of the compound are said to show wide variations in effectiveness. It is possible that chance has prevented such an observation with the present medium. Several different specimens of tellurite from diverse sources over a period of two years have shown entire uniformity of behavior. This cannot be accepted as evidence that other lots may not prove to be quite different, and one must keep this possibility in mind. The amount recommended in our formula was again chosen as a compromise. The objective was to permit the growth of as many colonies of *C. diphtheriae* from a weakly positive culture as would appear on a blood agar plate, and yet to inhibit the maximum number of contaminating organisms. It was desirable that growth of *C. diphtheriae* should be sufficiently rapid, in a strongly positive culture, to be readily obvious in 15 to 18 hours, and to show a satisfactory degree of characteristic darkening. The amount chosen is such that it has been possible, by simple inspection of the plate, to exclude as definitely negative the majority of throat cultures obtained in the Army survey. Swabs from the throats of children seem frequently to contain more micrococci which grow, although for the most part slowly, at this level of tellurite. The use of more tellurite—up to double the amount given in our formula—is permissible, and should remedy this difficulty, perhaps at the cost of missing an occasional very weak positive, such as a late convalescent case or healthy carrier. Lev, Pohl, and Tucker (1946) have simply doubled the amount of the “serum-tellurite” solution in making up plates and find the resulting medium more satisfactory.

The mechanism by which the tellurite functions is not clear. We have found that it is very considerably influenced by the presence of cystine. This amino acid, or sulfur in some similar form, is essential to the growth of those strains of *C. diphtheriae* which have been studied. The growth, especially of gravis strains, on our basic medium, *without tellurite*, is notably improved by the addition of cystine, which is present in hydrolyzed casein only in relatively small amounts. Unfortunately the addition of tellurite to plates containing additional cystine results in a very sharp inhibition of the growth of *C. diphtheriae* as well as of other organisms. It may well be that this observation, for which no explanation is apparent, may clarify, at least in part, the critical nature of the concentration of tellurite in other formulae, for in some instances cystine is added intentionally, whereas in others it may be present to a variable degree, depending on the protein used for the production of the peptone component.

The majority of formulae for tellurite media include whole blood, which is sometimes heated to obtain “chocolate” plates. It has been shown in earlier work from this laboratory (Cohen, Snyder, and Mueller, 1941) that the factors

influencing the growth of *C. diphtheriae* are present in serum and absent from red cells. It is, therefore, evident that a transparent and unheated serum medium will be quite as effective as one containing whole blood. One step (the reheating to "chocolate") is thus avoided in routine preparation; nonsterile collection of the serum followed by Berkefeld filtration provides suitable material, and a transparent plate, which many bacteriologists consider an advantage, results.

In the practical use of the medium, plates are poured and allowed to cool briefly without covers to avoid surface moisture. They will keep for considerable periods in the cold room. Each plate is marked off in sections with a wax pencil, and 6, 8, or even 12 swabs may be cultured on the standard petri dish, each swab being thoroughly smeared over a numbered section, but with care to avoid overlapping. After 15 to 18 hours' incubation the plates are examined. Easily visible confluent growth should be apparent in cultures from a fresh case infected with either the mitis or gravis type of *C. diphtheriae*. The presence of an intermedius strain offers certain difficulties which will be discussed below. Plates showing no growth are reincubated and again examined at 24 hours and finally 48 hours, since scattered colonies may escape attention on the first day. In 48 hours mitis colonies are 1.0 to 1.5 mm in diameter, black, and convex with a glistening surface. The gravis type is larger, 2 to 3 mm in diameter, flat with somewhat irregular edges (seldom typical "daisy-head"), and has a dull surface and a slate-gray color. The size of the colony and the degree of darkening of course increase with the time of incubation, but even in 18 hours with a strongly positive culture the difference in appearance of typical mitis and gravis growth is already detectable.

The intermedius type of *C. diphtheriae* seems to have been rather infrequently encountered or recognized. It is prevalent, in certain foci in Europe, Canada, and the United States, though accounting for only a small proportion of the total cases. The original description of the type by McLeod and his associates (Anderson *et al.*, 1933) together with its designation as "intermedius" seems to have caused some confusion. "Intermedius" clearly refers to the clinical severity, not to the cultural characteristics, of the organism. There is little doubt that the strains recently described by Frobisher, Adams, and Kuhns (1945) under the name of "minimus" are identical with those formerly classified by the British workers as "intermedius." Through the courtesy of Dr. Frobisher we have been able to compare directly his "minimus" strains with British cultures of "intermedius," kindly made available to us by Dr. Robert Cruikshank of the British Medical Research Council, and with Canadian strains, received through the kindness of Dr. E. T. Bynoe of the Laboratory of Hygiene, Ottawa. They appear alike, culturally and morphologically.

The intermedius colonies are quite different from the colonies of either gravis or mitis on the medium here described. They are relatively minute, pin point in size at 24 hours, and approximately 0.2 to 0.3 mm in diameter at 48 hours. Moreover, they show little darkening; although if the plate after 48 hours is laid on a sheet of white paper, in a good light, they are definitely brownish gray. With the colony microscope the appearance fits closely the description given by

Anderson, Cooper, Happold, and McLeod (1933), "fine flat colony with central knob and slightly crenated periphery." The detection of "intermedius" colonies on any tellurite medium, therefore, requires previous familiarity with the organism and a careful inspection of all cultures which at first glance appear negative. This is not so much a defect in tellurite plates as an unhappy characteristic of the organism in question, which grows but little better on blood agar.

Hoffman's bacillus and other less well characterized diphtheroids also grow on the tellurite medium here described. In appearance such colonies may closely resemble mitis diphtheria, or they may be less deeply colored and sometimes show a central dark spot surrounded by a gray-white periphery. Occasional cocci produce growth of the mitis type, and infrequently there develops a heavy growth of certain nondiphtheroid bacilli.

A considerable proportion of negative cultures can be discarded on the evidence of careful inspections of the plate after the intervals previously noted. When growth appears which is suggestive of diphtheria, a stained preparation should be examined. A quick glance at the preparation will enable the bacteriologist to discard a number of cultures from further consideration since they will be seen to be micrococci of one sort or another, yeast, or nondiphtheroid bacilli. The remainder, clearly corynebacterial in character, represent the potentially "positive" cultures.

Too much stress has been laid on the atypical morphology of *C. diphtheriae* on tellurite. The normal, well-nourished diphtheria bacillus probably has never been observed from Loeffler's medium, because the latter provides only a starvation diet. The rapidity and selectivity of its supposed growth-promoting properties are illusory. The diphtheria bacillus is an organism with relatively simple growth requirements that can multiply on almost any kind of nutrient medium, including Loeffler's, which, however, is extremely deficient for streptococci and pneumococci. One need only compare the colony of the diphtheria bacillus on Loeffler's medium and on a medium designed to supply maximal quantities of actual growth requirements, to appreciate this point. The latter type of colony can readily attain 100 times the area of the former. (See photographs—Cohen, Snyder, and Mueller, 1941.)

The morphology of the organism during the phase of rapid growth on an entirely favorable medium differs from its appearance when taken from Loeffler's medium, and both may differ from the morphology shown on tellurite. Which is "typical"? Of course the matter may be carried further. Loeffler's medium is sometimes prepared from beef serum, sometimes from that of hogs. The two behave quite differently—a matter to which allusion has already been made—and probably the morphological appearance may vary. In brief, morphology, as pointed out earlier, is an entirely subjective matter, and even so is dependent on a constant supply of a uniform and reproducible culture medium. In our experience the morphology on tellurite medium is entirely uniform. It varies with the type of *C. diphtheriae* in question, but is quite as readily learned as in the case of any other medium.

In any event, at this point the laboratory procedure must follow established

traditional—and purely objective—lines, if the decision is to be scientifically valid. The organism grown on the tellurite, or any other, medium must be tested for virulence or toxin production by one of the established methods.

The most complete identification, of course, consists in isolation of the organism by replating, followed by a study of its fermentative properties, hemolysis production, type of growth in broth, etc., and finally by a determination of its virulence in properly controlled guinea pigs or rabbits. It is permissible to hasten the virulence test by injecting an emulsion of the impure growth taken directly from the tellurite plate. With adequate control, such a procedure is even more satisfactory than when an original Loeffler's culture is used because of the relative freedom from other throat organisms. Such a method presents the further advantage of guarding against the concomitant presence of virulent and non-virulent strains of *C. diphtheriae* in the same throat—a state of affairs which may sometimes occur, and which could conceivably lead to a false negative report if isolation from a single colony were carried out.

Fermentation studies, after isolation of a pure culture, may be made on Hiss serum water (horse or beef serum) containing glucose, sucrose, and starch. The ability to ferment the latter is one of the most characteristic features of the gravis type. A word of caution on its use, however, may not be out of place. Starch, on hydrolysis, yields glucose, which is promptly fermented by all strains of *C. diphtheriae* as well as by many other diphtheroids. Since native starch is insoluble in cold water, commercial preparations called "soluble starch" have obviously been subjected to some type of alteration, which may include moderate hydrolysis with the production of variable amounts of glucose. Their use is best avoided, and one should employ corn starch or laundry starch. This is readily incorporated into the medium by adding it to the cold serum water and heating to boiling while stirring to prevent the formation of lumps. Alternatively, a 5 per cent starch paste is prepared by pouring a heavy suspension of starch in cold water into a suitable volume of actively boiling water, stirring vigorously. This solution is then used in preparing the serum water.

Fermentations by the members of the diphtheria group are usually prompt, clear-cut, and typical. As in the case of other organisms, the fact that irregularities may occur should be kept in mind. The prime requisite for a "positive" fermentation is that good growth should occur, and tubes failing to show the expected result should be examined from this standpoint. The use of the old but very excellent Hiss serum water goes far toward insuring prompt growth. The presence in the serum of certain still-unknown growth-promoting factors, which are heat-stable, probably accounts for this fact. Particularly in the case of intermedius strains, one must take care to inoculate heavily into a really suitable medium and examine for the presence of growth after incubation if failure to ferment typically is noted.

In concluding, it may not be out of place to refer briefly to the purpose of the laboratory examination for the diphtheria bacillus and to how much the clinician should expect from it. The laboratory cannot "diagnose" diphtheria—that is the function of the physician. The bacteriologist may be able to state, following

a delay of 12 to 15 hours, that organisms which he believes are consistent in morphology with *C. diphtheriae* are present in his culture. If he has had long practical experience in the matter, he may be able to make a similarly tentative statement even sooner, by examination of a direct smear made from the throat swab, but such an opinion is best not ventured by the inexperienced. Moreover, the failure to observe the organism in early, or even later, culture by no means excludes diphtheria in the patient. An improperly taken throat swab may yield entirely negative results, although more careful subsequent culturing may show the organism to be present abundantly in certain areas.

Under optimal conditions, the laboratory can report after from 2 to 4 days that a virulent diphtheria bacillus has been obtained from the culture. This does not of itself establish a clinical diagnosis of diphtheria, for the condition may have occurred in the throat of an immune carrier and may have been entirely non-diphtherial in nature. The decision as to the initial diagnosis and treatment of the case is the direct and immediate responsibility of the physician. Even a few hours' delay in administration of antitoxin may result in an unfavorable outcome, and, in general, such specific treatment should be instituted on suspicion sufficiently strong to persuade the clinician to seek laboratory confirmation. The occasional needless use of antitoxin will, in general, do less harm than the delay entailed by waiting for a laboratory report before its administration. In the very mild or atypical case an early presumptive laboratory report may be of assistance in establishing a correct diagnosis, but, in general, it is the function of the bacteriologist to confirm or disprove a clinical decision and thus to provide the basis for suitable later handling of the case in question, and to establish a background which may serve to guide future clinical judgment.

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A MEDIUM FOR THE NAGLER PLATE REACTIONS FOR THE IDENTIFICATION OF CERTAIN CLOSTRIDIA

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Hayward (1941, 1943) developed a plate reaction for the rapid identification of *Clostridium perfringens* (*C. welchii*) based upon the so-called Nagler reaction. This reaction involves the splitting by certain toxins of insoluble fatty material from human blood serum or egg yolk, and the appearance of a zone of precipitation around positive colonies in plate cultures. The medium recommended by Hayward (1943) was nutrient agar containing 20 per cent human serum and 5 per cent peptic digest of sheep's blood. For the identification of *Clostridium oedematiens* (*C. novyi*) Nagler (1944a, 1944b) used Weinberg's V. F. agar (peptic digest of beef liver, etc.) with 10 per cent defibrinated sheep's blood and 10 per cent egg yolk suspension.

We have investigated these reactions, using a large number of each of these species and several others. One of our objectives has been to devise a medium on which the reaction could be demonstrated that could be prepared from easily available materials. Several media which appear to give promise will be discussed in later publications. The following is recommended as a satisfactory substitute for the media suggested by Hayward and by Nagler:

Proteose peptone no. 2.....	40 g
Na ₂ HPO ₄	5
KH ₂ PO ₄	1
NaCl	2
MgSO ₄	0.1
Glucose.....	2
Agar.....	25
Distilled water.....	1,000 ml
pH.....	7.6
Sterilize: 240 F for 20 minutes	

After autoclaving, add 10 ml of sterile egg yolk suspension to 100 ml of warm medium and pour approximately 15 ml in plates of 100-mm diameter. The addition of the blood to the medium, as suggested by Nagler, is unnecessary. To prepare egg yolk suspension aseptically, withdraw to a sterile rubber-stoppered tube by aspiration the yolk from a fresh hen's egg after first removing the white. Add an equal amount of sterile 0.85 per cent NaCl to the yolk and invert the tube to mix the contents.

We shall describe in subsequent papers the characteristic differentiating reactions, which may be inhibited by appropriate specific antiserum, obtained with streaked plate colonies of each of the following members of the genus *Clostridium*: *C. perfringens* (*C. welchii*), *C. oedematiens* (*C. novyi*) types A and B, the *C.*

oedematoides-*C. sordelli*-*C. bifermentans* group, *C. parabotulinum* types A and B, *C. botulinum* types B, C, D, and E, and *C. sporogenes*. The following species have failed to give reactions: *C. tetani*, *C. histolyticum*, *C. tertium*, *C. septicum*, *C. capitovalis*, *C. chauwoei*, and *C. cochlearium*.

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METABOLISM AND THE CHEMICAL NATURE OF STREPTOMYCES GRISEUS¹

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Although considerable information has accumulated concerning the distribution of actinomycetes in nature and their cultural characteristics, comparatively little is known of the chemical composition of their mycelium, their physiology, and their nutrient requirements. With the growing importance of actinomycetes as producers of antibiotic substances, it seemed desirable to gain some information concerning the composition of their cells and the chemical changes that they bring about in the composition of the medium. Certain pertinent data obtained in the study of the streptomycin-producing organism, *Streptomyces griseus*, may therefore be of interest.

Since the organism is commonly grown either in stationary cultures or in a submerged and agitated state, a comparison was first made of the rate of cell synthesis of the organism, the consumption of some of the nutrients, and the degradation of certain of the organic constituents in the medium under these two cultural conditions.

Nutrient broth containing 5 g peptone, 5 g meat extract, 10 g glucose, and 5 g NaCl per liter was used as the stock medium. For stationary cultures 250-ml portions of the medium were placed in 1-liter Erlenmeyer flasks, sterilized, and inoculated with a spore suspension of the organism. The cultures were incubated at 28 C, and, at various intervals, several of the flasks were removed for analysis. The mass of growth including the mycelium and the spores was filtered on weighed papers, dried at 65 C, and weighed. Some of the pellicles were analyzed for ash and others for total nitrogen. The culture filtrates were analyzed for antibiotic potency, as well as for ammonia and amino nitrogen (tables 1 and 2).

In stationary cultures the growth of *S. griseus* reached a maximum in 10 days and then gradually diminished as a result of slow lysis of the cell material. The ash and nitrogen content of the mycelium tended to be higher during the early stages of growth, and to decrease after growth had reached a maximum. The amino nitrogen in the culture gradually increased to nearly double that present in the original medium, reaching a maximum in 5 to 7 days, and then remaining at a somewhat constant level. The ammonia increased from a trace to nearly a half of the total nitrogen in the culture after 15 days.

Streptomycin production began in stationary cultures only after about 5 days' incubation, when the amino nitrogen reached its high level. The maximum concentration of streptomycin corresponded to the peak of growth of the organism,

¹ Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

as determined by weight and nitrogen content. Considerable variation was obtained in the weight of the pellicles obtained from different flasks. In order to determine to what extent this variation affects the chemical composition of the mycelium, several heavy and light pellicles were analyzed. Comparatively little difference was obtained in the percentages of nitrogen and ash in such pellicles.

In the submerged cultures (table 3) growth of the organism was more rapid and more abundant; the maximum was reached in 3 to 5 days and was followed

TABLE 1
Rate of growth and streptomycin production of S. griseus in stationary cultures
Per 250-ml portions of medium

INCUBATION	GROWTH	STREPTOMYCIN
<i>days</i>	<i>g</i>	<i>units/ml</i>
4	0.364	<5
5	0.437	8
7	0.449	13
10	0.695	128
15	0.640	140
21	0.507	125

TABLE 2
Nitrogen fractions in S. griseus cultures
Per 250-ml portions of medium

INCUBATION	NITROGEN IN MYCELIUM		NH ₃ -N IN BROTH	NH ₃ -N IN BROTH	TOTAL N* ACCOUNTED FOR	ASH CONTENT
<i>days</i>	<i>per cent</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent</i>
0			4.3	35.3		
4	10.0	35.2	22.6	57.3	115.1	12.1
5	9.7	40.3	37.8	69.5	147.6	16.4
7	10.0	55.8	55.9	73.3	185.0	14.6
10	8.9	62.4	63.3	66.8	192.5	13.9
15	9.6	55.2	92.6	79.3	227.1	10.0
21	7.2	37.6	95.1	70.8	203.5	11.9

* The original broth contained 280.0 mg total nitrogen.

by a more rapid lysis. The more extensive growth was also accompanied by a more rapid consumption of the sugar and by a rise in pH value and in production of streptomycin. Lysis of the culture, as seen by a reduction in weight of the mycelium, was accompanied by a loss in streptomycin activity. It is not known as yet what interrelation, if any, exists between these two phenomena. Most important in this connection is the fact that streptomycin, a substance highly resistant to the action of microorganisms and enzymes, is gradually inactivated in its own culture medium. The nature of the mechanism involved remains to be elucidated. The fact that the reaction of the medium is changed from pH 6.8

to 8.6 may have a favorable effect upon the production of streptomycin, but whether it has a bearing upon its destruction is yet to be determined.

It will be recalled that the nonsporulating and inactive strains of the organism do not bring about any extensive alkalinity in the medium, such strains undergoing much lysis, especially in submerged cultures. These strains actually give rise to an initial acidity of the medium, followed by a gradual change to an alkaline reaction (Schatz and Waksman, 1945).

A study of the organic composition of the mycelium of an active strain of *S. griseus* was now made by the use of various solvents. Twelve-day-old

TABLE 3
Rate of growth of S. griseus and streptomycin production in shaken cultures

INCUBATION	GROWTH	pH OF FILTRATE	RESIDUAL GLUCOSE	STREPTOMYCIN
<i>days</i>	<i>g</i>		<i>mg/ml</i>	<i>units/ml</i>
0		6.8	10.2	0
1	0.048	6.9	9.3	<5
2	0.237	8.5	7.6	5
3	0.394	8.6	5.6	63
5	0.370	8.4	0.5	84
7	0.248	8.7	0.5	62
10	0.140	8.9	0.5	51

TABLE 4
Chemical nature of the mycelium of S. griseus

FRACTION	YIELD	PER CENT OF TOTAL	ANTIBIOTIC ACTIVITY OF 1 MG OF MATERIAL		
			<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>
	<i>g</i>				
Ether-alcohol*	1.84	16.3	<1	30	90
Chloroform	0.11	1.0	<5	5	50
Cold water	4.18	37.1	14		120
Hot water	1.02	9.0			
Residue	4.12	36.6			
Total	11.27	100.0			

* The ether-soluble fraction of this extract was 0.71 g.

pellicles from surface cultures of a total of 10 liters of medium were collected, washed with distilled water, and allowed to drain. The mycelium was extracted first with a mixture of 50 per cent ether and 50 per cent alcohol. This was followed by extraction with chloroform, then with cold water, and finally with hot water for 1 hour on a boiling water bath. The various fractions, as well as the residual material, were dried and weighed. The results (table 4) show that more than a third of the constituents of the mycelium are soluble in cold water, and another third or more is found in the residual fraction, insoluble in organic solvents and in cold and hot water. The water-soluble fraction was different in composition from the insoluble fraction; the total nitrogen of the first was 10.7

per cent, as compared with 6.1 per cent of the second. When the insoluble residue was treated with 2 per cent HCl for 2 hours on a water bath, about 70 per cent of it was hydrolyzed; 17 per cent of the total residue was accounted for as reducing sugar. The hot-water-soluble fraction was highest in nitrogen, giving 13.7 per cent on a dry basis and 15.5 per cent on an ash-free basis, thus approaching pure protein.

Several of the extracts were tested for antibiotic activity. Only the antibacterial spectrum of the cold-water-soluble fraction appeared similar to that of typical streptomycin; the total numbers of *Escherichia coli* and *Bacillus subtilis* units of this fraction are reported. The ether-alcohol and chloroform extracts had no detectable activity against *E. coli*; they were, however, very active against gram-positive bacteria. This suggests the probability that the mycelium contains another antibiotic substance which is different from the typical streptomycin, as already reported elsewhere (Schatz and Waksman, 1944). This other fraction is not active against *E. coli*, but it is active on *B. subtilis* and other gram-positive bacteria.

TABLE 5

S. griseus pellicle as a source of precursor for streptomycin production
Stationary cultures—NaCl and glucose stock solution

TREATMENT OF MEDIUM	STREPTOMYCIN PRODUCED, UNITS PER 1 ML		
	6 days	8 days	11 days
<i>S. griseus</i> pellicle.....	4	7	30
<i>S. griseus</i> pellicle + 5 g peptone.....	26	36	115
Normal medium (peptone + meat extract).....	37	50	173

Since the presence in the medium of a specific organic constituent, similar to that found in meat extract or corn steep (Schatz, Bugie, and Waksman, 1944), is required for the production of streptomycin, the question arose whether *S. griseus* is capable of synthesizing a compound similar to that found in meat extract, which may be of the nature of a precursor or a substance essential to the mechanism responsible for the synthesis of streptomycin.

Pellicles of *S. griseus* from given volumes of medium were added to similar lots of media containing only 5 per cent NaCl and 1 per cent glucose; the mycelium of the organism was thus made to serve as a source of both nitrogen (peptone) and the meat extract factor of the regular medium. Peptone was added to other flasks, so that the mycelium served only as a substitute for meat extract. The results reported in table 5 show that whereas the mycelium of *S. griseus* cannot take the place of both peptone and meat extract, it can well replace the latter as a "precursor" of streptomycin or of one of the essentials in the production mechanism. Dried fungous mycelium (*Aspergillus niger*) could not serve as the source of the "precursor"; however, yeast preparations and yeast extracts were highly efficient, but acid-hydrolyzed yeast was not.

In order to determine whether only mycelium of active strains of *S. griseus* can serve as the essential "precursor" for streptomycin production, the following experiment was conducted. Seven-day-old pellicles of an inactive strain of *S. griseus* and of an active, streptomycin-producing strain were grown in two media, one containing meat extract and the other free of it. The pellicles were washed with tap water, added to comparable volumes of fresh media free from meat extract, sterilized, and inoculated. The results (table 6) show that the streptomycin "precursor" is actually synthesized by *S. griseus*, whether or not an active streptomycin-producing strain is used, and whether or not the pellicle is produced on a medium favorable for streptomycin production.

S. griseus produces small amounts of streptomycin even in the absence of meat extract, thus demonstrating that the organism is capable of synthesizing the streptomycin factor. Further, the fact that the mycelium of an inactive strain of *S. griseus* was as efficient in producing the streptomycin factor as was the active strain pointed to a general capacity of the organisms to synthesize the factor independent of the ability to produce streptomycin itself.

TABLE 6

The efficiency of pellicles of different S. griseus strains as precursors for streptomycin
Stationary cultures

SOURCE OF PRECURSOR	MEAT EXTRACT PRESENT IN ORIGINAL MEDIUM IN WHICH PELLICLE WAS GROWN	ACTIVITY, UNITS PER ML, 8 DAYS
<i>S. griseus</i> 3378*.....	+	168
<i>S. griseus</i> 19.....	0	158
<i>S. griseus</i> 19.....	+	163
Meat extract (control medium).....		182

* Inactive strain.

The essential factor provided by meat extract or by the mycelium of the organism may be either a precursor of the whole or part of the streptomycin molecule or a prosthetic group in an enzyme system essential for streptomycin production. The results of various experiments not reported here indicate that once a pellicle of the streptomycin-producing strain of *S. griseus* has been produced, several successive replacements using a simple glucose or glycerol nitrogen (glycine or NaNO_3) solution containing inorganic salts will yield filtrates of approximately the same order of activity. Such findings would tend to give support to the second hypothesis. If the meat extract provides a precursor of streptomycin, the activity of each successive lot of the replaced synthetic medium would tend to decrease as the precursor in the pellicle became exhausted, unless one postulates the reformation of the precursor. The maintenance of fair activity in the repeatedly replaced synthetic solutions indicates no exhaustion of precursor, but rather that the essential factor functions in the streptomycin-producing mechanism; once it is present in the mycelium, it is retained and continues to function for a long time.

It is proposed to designate the specific substance required for maximum production of an antibiotic agent as the "activity factor," in contradistinction to the accepted term "growth factor" essential for maximum cell synthesis. The activity factor is not necessary for normal growth of the organism, since *S. griseus* will grow equally well on a variety of media with or without meat extract.

By definition, a growth factor is a substance required in very minute amounts for optimum growth of an organism. However, many organisms, such as certain bacteria and fungi that require specific preformed organic substances, are able to make limited growth in the absence of such factors. The production of streptomycin represents a somewhat analogous phenomenon: without the meat extract factor, *S. griseus* forms streptomycin, but in relatively small amounts; when meat extract is present in the medium, *S. griseus* produces a highly active antibiotic filtrate. The meat extract which allows maximum streptomycin production thus provides for *S. griseus*, not a growth factor, but an activity factor. Because of the complex chemical nature of streptomycin

TABLE 7
Antibiotic spectrum of the second antibiotic substance of S. griseus

TEST ORGANISM	GROWTH INHIBITION
	<i>units/gram</i>
<i>Escherichia coli</i>	<100
<i>Pseudomonas aeruginosa</i>	<100
<i>Bacillus mycoides</i>	320,000
<i>Bacillus subtilis</i>	800,000
<i>Mycobacterium phlei</i>	210,000
<i>Mycobacterium tuberculosis</i> (H37).....	<3,200
<i>Mycobacterium avium</i>	11,000

(Brink *et al.*, 1945) it is still difficult to say what the function of the "factor" may be.

It has been reported elsewhere (Schatz and Waksman, 1944) that *S. griseus* produces another antibiotic substance which is distinct from typical streptomycin. Small amounts of this substance can be extracted from the culture filtrate with ether. Much larger quantities, however, can be obtained by the extraction of the mycelium with acetone or ether. Upon removal of the solvent, the residue is taken up in 95 per cent ethyl alcohol. It is to be recalled that streptomycin is insoluble in organic solvents.

A typical antibacterial spectrum of the second substance is shown in table 7. This spectrum shows the substance to be markedly different from streptomycin, which is active against gram-negative bacteria and against *Mycobacterium tuberculosis* var. *hominis*. It is interesting to note that the second factor is more active against the avian strain than against the human—a condition which is the reverse of that shown by streptomycin. There appears to be no correlation between the production of streptomycin and the production of the second antibiotic factor.

The ability of various organisms to produce more than one antibiotic factor is common, as illustrated by the ability of *Bacillus brevis* to form gramicidin and tyrocidine, and of *Penicillium notatum* to produce penicillin (in several forms) and notatin. *Aspergillus fumigatus* actually produces four antibiotics: fumigatin, spinulosin, fumigacin, and gliotoxin.

SUMMARY

The growth of *Streptomyces griseus* reaches a maximum in stationary cultures in 10 days and in submerged cultures in 3 to 5 days, followed by the lysis of the mycelium. Growth is accompanied by a gradual rise in the pH value of the culture, and in the ammonia and amino nitrogen contents. The total nitrogen in the mycelium tends to be higher during the active stages of growth. The production and accumulation of streptomycin parallels the growth of the organism. After maximum activity has been reached, there is a drop in activity, which is rapid in submerged cultures. For the production of streptomycin, the presence in the medium of an organic substance is required. This substance may be designated as an "activity factor," either serving as the precursor of the streptomycin molecule as a whole or of an important group in the molecule, or functioning as a prosthetic group in the mechanism essential for the synthesis of the streptomycin. Such a factor can gradually be synthesized by the organism. When it is provided in the medium in a preformed state, however, as in meat extract or in corn steep, the process of streptomycin synthesis is greatly facilitated.

Streptomyces griseus produces another antibiotic substance which is present in a limited amount in the culture filtrate but more abundantly in the mycelium. This second substance is distinct from streptomycin, since it is soluble in organic solvents and is not active against gram-negative bacteria.

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THE PRODUCTION OF PENICILLIN X IN SUBMERGED CULTURE

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It is now generally known that members of the *Penicillium notatum-chrysogenum* group are able to produce at least four different penicillins, known as F, G, X, and K,² when grown under the usual conditions of laboratory or commercial culture. It is likewise recognized that two or more of these penicillins may occur in the same culture broth, the proportions being influenced to a considerable degree by the particular strain used and the specific cultural conditions employed. Since these penicillins differ in chemical characteristics and in their inhibitory effect upon susceptible bacteria, it has long seemed reasonable that they might possess different possibilities in clinical application. The first clinical evidence in support of this possibility was provided by Welch, Putnam, Randall, and Herwick (1944). They reported that penicillin X *in vitro* was more effective than commercial penicillin (primarily penicillin G) against a strain of *Klebsiella pneumoniae* type A and *Bacillus cereus*, and that it was three to five times as effective as commercial penicillin in protecting mice against 10,000 lethal doses of pneumococcus type I. The same investigators found penicillin X to offer particular promise in the treatment of gonorrhea: bacteriological cures were obtained in 64 of 68 cases (94 per cent), mostly sulfonamide-resistant, which were treated with single 25,000-unit intramuscular injections; whereas only 37 cures were obtained in 58 comparable cases (64 per cent) treated with single 25,000-unit intramuscular injections of commercial penicillin. They further observed that patients given penicillin X maintained a consistently higher blood concentration, and that this penicillin was excreted into the urine more slowly than commercial penicillin. A more recent report by Ory, Meads, and Finland (1945) confirms the earlier work of Welch *et al.* (1944). Group A hemolytic streptococcus, gonococcus, and meningococcus were found to be from two to eight times more sensitive to preparations containing 65 or more per cent of penicillin X than to commercial penicillin (primarily penicillin G), whereas most strains of pneumococcus and *Streptococcus viridans* were twice as sensitive to penicillin X. As reported by Welch *et al.* (1944), staphylococci were found to be equally sensitive to the two kinds of penicillin. Levels of penicillin activity in the serum were significantly higher and were sustained longer after intramuscular injections of penicillin X than after injections of the same number of units of commercial penicillin. Subsequent to that work, Libby and Holmberg (1945) found penicillin X to be more effective than penicillin G against a number of different bacteria including various

¹ This is one of four regional laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

² These penicillins are known as I, II, III, and IV (?), respectively, in the United Kingdom (Coghill and Koch, 1945).

streptococci and pneumococci, and Flippin and associates (1945) reported the successful use of penicillin X in the treatment of a case of bacterial endocarditis (*Streptococcus viridans*) which had failed to respond to repeated administrations of commercial penicillin.

Realizing that the different penicillins might have special applications, members of the Fermentation Division had given some attention to the development of culture conditions favoring the production of particular penicillins before the appearance of the paper by Welch *et al.* (1944), but no concerted attempt had been made to select, isolate, or develop an organism characterized by the production of increased amounts of a particular penicillin as opposed to an increase in total yield. Because of the possible important clinical role of penicillin X, and because of its interesting chemical possibilities, such a course now seemed warranted.

The presence of penicillin X was observed first in surface cultures of NRRL 1249.B21, a derivative of the Fleming strain (Raper, Alexander, and Coghill, 1944; Raper and Alexander, 1945; and Moyer and Coghill, 1946a); and the materials employed by Welch *et al.* (1944) and Ory and associates (1945) were undoubtedly of this origin. Surface culture trials made at this laboratory showed this strain to be the best penicillin X producer among ten selected cultures with maximum total yields of 155 to 160 units per ml, of which 50 to 57 units per ml, or about 30 to 35 per cent, represented penicillin X. Although this ratio was very promising, it was realized that if penicillin X were to be made in quantity, some strain capable of producing substantial amounts in submerged culture would be required, and no derivative of the Fleming culture had ever been found satisfactory for this method of production. Attention was thus directed toward good "submerged" strains. Differential assays (Schmidt, Ward, and Coghill, 1945; and Schmidt, 1946) were made upon samples taken from rotary drum and vat fermentations then being conducted by Dr. G. E. Ward and associates (unpublished data). No detectable amount of penicillin X was found in cultures of *Penicillium chrysogenum* NRRL 1951.B25, or either of three substrains derived from it, including X-1612, the highest known producer of penicillin in submerged culture. However, yields of this penicillin amounting to somewhat more than 15 per cent of the total were produced by *P. chrysogenum* NRRL 1984.A. With this background, the writers set about in March, 1945, to secure, if possible, a substrain of 1984.A capable of producing substantially increased ratios of penicillin X. By the end of August such a strain had been developed and its performance repeatedly verified. The present report is concerned with this development.

MATERIALS AND METHODS

Molds included in study. A considerable number of different cultures were investigated during the present study. To individuals generally familiar with the whole penicillin development in this country, the origins and numerical designations of these strains will be commonplace. In the belief that some information regarding the source and known potentialities of these cultures will

prove of interest and value to other readers, a somewhat detailed list is presented.

NRRL 1249.B21 *Penicillium notatum*, derived from the Fleming culture (Raper and Alexander, 1945), a substrain developed at the Northern Regional Research Laboratory which produced high yields of penicillin in surface culture (Raper, Alexander, and Coghill, 1944, and Moyer and Coghill, 1946a). This strain has been used in industry more than any other for the surface production of penicillin in this country and abroad.

NRRL 832.A2 *Penicillium notatum*, a substrain of NRRL 832 which normally produces somewhat higher yields of penicillin than the parent (Raper and Alexander, 1945). The latter culture was the first mold found to produce good yields of penicillin in submerged culture (Raper, Alexander, and Coghill, 1944; Moyer and Coghill, 1946b), and was generally employed in industry for this type of production during the period prior to the summer of 1944. Maximum yields of about 100 units per ml have been obtained.

NRRL 1951.B25 *Penicillium chrysogenum*, a substrain developed at the Northern Regional Research Laboratory from NRRL 1951 (isolated from a cantaloupe collected in Peoria, Illinois), which produced substantially greater amounts of penicillin in submerged culture than NRRL 832 and substrains derived therefrom (Raper and Alexander, 1945). Yields in excess of 200 units per ml have been obtained in large-scale laboratory apparatus. This culture was widely used in industry for submerged production during the latter half of 1944 and the early months of 1945. It found limited use for surface production, being less sensitive to temperatures in excess of 24 C than NRRL 1249.B21. It was selected as a promising strain for X-ray and ultraviolet irradiation, and a number of noteworthy substrains were developed from it (see below).

Stanford 25099 *Penicillium chrysogenum*, a substrain of NRRL 1951.B25 resulting from conidia exposed to ultraviolet radiation, distributed by Professor G. W. Beadle and associates as a superior submerged culture and studied in many laboratories.

Stanford 35217 *Penicillium chrysogenum*, a substrain of NRRL 1951.B25 resulting from conidia exposed to ultraviolet radiation, distributed by Professor Beadle and associates as a superior submerged culture. As studied in many laboratories, this substrain generally produced somewhat better yields than the parent culture, and it has found limited use in industry.

X-1612 *Penicillium chrysogenum*, an X-ray-induced mutation of NRRL 1951.B25, produced by Dr. M. Demerec and associates at the Carnegie Institute of Washington, Cold Spring Harbor, New York, tested by Professor C. M. Christensen and associates at the University of Minnesota, and forwarded to Professors W. H. Peterson and M. J. Johnson of the University of Wisconsin as a superior strain. When tested in 80-gallon fermenters by the latter investigators, this mutant was found to be an exceptionally good penicillin producer, and yields in excess of 500 units per ml were obtained (unpublished data). The strain was subsequently tested at the Northern Regional Research Laboratory (with yields up to 450 units per ml) and other laboratories, and its superior qualities were confirmed.

NRRL 1984 *Penicillium chrysogenum*, received as Minn. R-13 from Professor C. M. Christensen of the University of Minnesota where it was isolated from Minnesota soil. The culture was recommended for submerged production and represents the parent of commercially important strains.

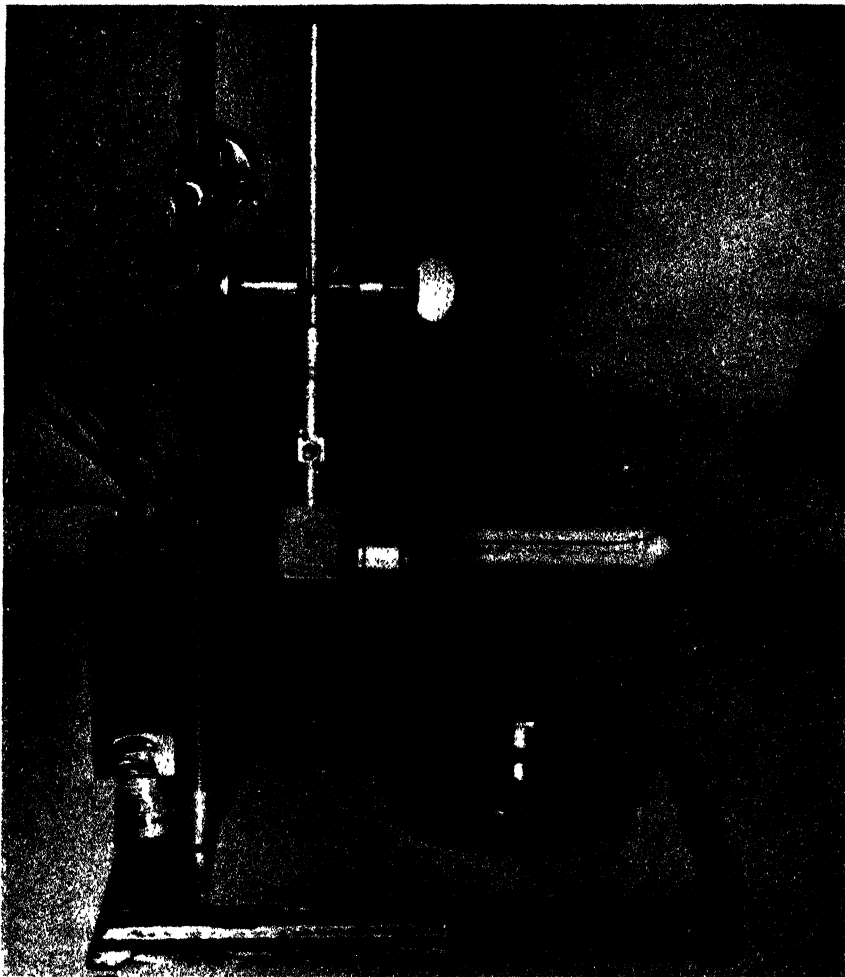


FIG. 1. APPARATUS AND TECHNIQUE EMPLOYED FOR THE IRRADIATION OF *PENICILLIUM* SPORES

Minn. 9SS251 *Penicillium chrysogenum*, a substrain of Minn. R-13, of monospore origin, selected at the University of Minnesota and distributed as a high-yielding submerged culture.

NRRL 1984.A *Penicillium chrysogenum*, a substrain of NRRL 1984 (Minn. R-13) selected at the Northern Regional Research Laboratory and found in repeated trials to produce somewhat greater yields than the parent. This strain has been intensively studied at the NRRL and is successfully used in industry. Yields in shaken flasks up to 150 to 160 units per ml are not uncom-

mon, and yields up to 260 units per ml have been obtained in rotary drum fermenters.

NRRL 1984.N22 *Penicillium chrysogenum*, a substrain of NRRL 1984.A resulting from conidia exposed to ultraviolet radiation. This strain produces good submerged yields, of which approximately half the total units present, as assayed with *Staphylococcus aureus*, represent penicillin X. The present paper is largely concerned with the development of this strain.

NRRL 1975.B *Penicillium chrysogenum*, a substrain selected at the Northern Regional Research Laboratory from a culture isolated from Nebraska soil at the University of Minnesota and sent to us by Professor C. M. Christensen as Minn. R-123. Yields up to 170 to 175 units per ml have been obtained in shaken flasks and approximately 250 units per ml in rotary fermenters, but performance is not so consistent as NRRL 1984.A.

Irradiation technique. A 4-watt General Electric germicidal lamp, of bent-tube construction, emitting 500 milliwatts of 2,537 Å (95 per cent of the total radiation), was employed as a source of ultraviolet light (figure 1). The lamp was supported on a ring stand in a horizontal position at either 2 or 4 inches above the base. The spores (conidia) to be irradiated were thoroughly dispersed in sterile water containing sodium lauryl sulfonate in a concentration of 1 to 10,000 as a wetting agent. During the irradiation the suspension was contained, to a depth of approximately 2 to 3 mm, in a flat-bottomed, uncovered petri dish placed on the base of the ring stand. The suspension was continuously stirred by means of a bent glass rod throughout the whole period of irradiation to insure as uniform exposure as possible. One-ml samples were removed from the suspension at 0, 1, 2, 4, 6, 8, 12, and 16 minutes, the greater portion of the total suspension being removed in the eight samples. Appropriate dilutions were made and the samples plated in Czapek's solution agar. Viability counts were made at 4 or 5 days, and random isolations were made from developing colonies from selected exposures. The cultures thus obtained were then tested in shaken-flask culture for their capacity to produce penicillin X when grown submerged.

Culture conditions. The following standardized culture solution, essentially like that previously employed for testing new isolates or variants (Moyer and Coghill, 1946b; Raper, Alexander, and Coghill, 1944; and Raper and Alexander, 1945) for their capacity to produce penicillin in submerged cultures, was employed:

Lactose.....	20.0 g
MgSO ₄ ·7H ₂ O.....	9.25 g
KH ₂ PO ₄	0.5 g
NaNO ₃	3.0 g
Concentrated corn steeping liquor...	40.0 ml
Cerelose (commercial glucose).....	1.2 g
Distilled water.....	1.0 L

Cultures were grown in cotton-stoppered, 1-liter Erlenmeyer flasks containing 200-ml quantities of culture solution, to each of which was added 1.6 g of sterile CaCO₃ and two drops of sterile lard oil before inoculation. Cultures were seeded

with spore suspensions washed from 5- to 6-day-old cultures, and a quantity sufficient to contain approximately 20 to 40 million spores was added to each flask. Cultures were tested in duplicate or triplicate flasks depending upon the assay facilities available. Incubation was at 24 to 25 C. Continuous agitation was effected by incubation on a rocker-type shaker making 100 three-inch strokes per minute.

Chloroform extraction. Penicillin X was isolated in pure form in one large-scale fermentation conducted at the end of this investigation. In all other experiments, the presence of this penicillin has been presumed when (1) an appreciable fraction of the total penicillin was not extracted by chloroform at pH 2.0 to 2.5, and (2) the unextracted penicillin gave a high assay ratio (see below) when tested against *Bacillus subtilis* (rough) and *Staphylococcus aureus*. The extraction technique³ was as follows: Add 5.0 ml of broth to 5.1 ml of purified chloroform, cool in an ice bath, and add 1:1 phosphoric acid to pH 2.0 to 2.5. Two to five drops are usually required. The quantity is determined on a separate portion. Shake vigorously and centrifuge about 2 minutes in a chilled centrifuge head. Replace in the ice bath and at once pipette 0.5 ml of the supernatant aqueous layer into 9.5 ml of buffer at pH 7.0. This may be used for assay, or diluted further with pH 6.0 buffer. The chloroform is washed, before use, with NaHCO₃, followed by two washings with water. The buffer, pH 7.0, consists of K₂HPO₄, 22.2 g, and KH₂PO₄, 7.8 g, dissolved and made up to one liter.

Assays. Assays of total penicillin were usually made daily on the third through the seventh days, but differential assays of chloroform-insoluble penicillin were usually made on the fourth, fifth, and sixth days only. Assays were performed by William H. Schmidt and assistants, and were made by the cylinder-plate method as reported by Schmidt and Moyer (1944), and as later improved by Schmidt (1946). Total penicillin was determined by assaying the untreated culture broth against *Staphylococcus aureus*, NRRL B-313 (F.D.A. no. 209P). The amount of chloroform-insoluble penicillin, presumably penicillin X, was determined by assaying an extracted broth against both *Staphylococcus aureus*, B-313, and the rough phase of *Bacillus subtilis*, NRRL B-558. As reported by Schmidt, Ward, and Coghill (1945), and by Schmidt (1946), the differential assay is based upon the varied responses of these organisms to penicillin X. Pure penicillin X assays approximately 950 units per mg against *S. aureus*, B-313, when pure penicillin G at 1,667 units per mg is used as a standard; against the same standard, its assay value varies from 1,200 to 1,900 units per mg against *Bacillus subtilis*, B-558(R), depending upon the phase of the test organism. The penicillin present in an extracted broth is presumed to be primarily penicillin X if this broth shows a differential assay ratio⁴ which corresponds closely to the assay ratio for the pure penicillin X standard for the same day. Assay ratios

³ Chloroform extractions were performed by Z. Louise Smith and G. E. N. Nelson under the supervision of Drs. G. E. Ward and R. G. Benedict.

⁴ The assay ratio for a given sample is defined as the assay value shown by that sample toward *Bacillus subtilis* NRRL B-558(R), divided by the assay value shown by the same sample toward *Staphylococcus aureus* NRRL B-313. Pure penicillin G is used as the standard in both cases.

fluctuate from day to day (table 1), increasing with an increase in the degree of roughness of *B. subtilis*, B-558. Ratios between 1.3 and 2.0 may be regarded as satisfactory, although the upper half of this range is preferred.

Total yields of penicillin and yields of penicillin X as shown in the figures and as cited in the text refer in all cases to values obtained with *S. aureus*, B-313. The assay values obtained with B-558(R), when interpreted in relation to the pure penicillin X assay ratio for the same day, are useful primarily as a check on the identity of the penicillin remaining in the extracted broth. For example, if the assay ratio is approximately 2.0 for a particular day, and the B-313 assay values in penicillin units are approximately one-half the units obtained with B-558(R) for a particular sample, then it can be assumed that the extraction has been satisfactory, and that the penicillin being measured is primarily penicillin X. If, however, the assay ratio is approximately 2.0, but the B-313 and the B-558(R) assays are approximately equal, then the active material present cannot be regarded as containing an appreciable amount of penicillin X, and one can usually assume that the chloroform extraction was poor. It is not believed that any useful purpose would be served by listing the B-558(R) assays generally, or in attempting to show them in the graphs. In table 1, where comparisons are made between penicillin X production by NRRL 1984.A and NRRL 1984.N22, the ultraviolet-induced mutation produced from it, the general relationships between B-313 assays of total yields, and B-313 and B-558(R) assays on chloroform extracts for a selected group of samples are shown. The agreement that can be expected between assay ratios for chloroform extracts and for pure penicillin X, on the same days, is also indicated.

EXPERIMENTAL

Preliminary trials. Although the highest yielding "submerged" cultures had been included in Ward's tests, the writers felt that, in seeking better penicillin-X-producing strains, some preliminary trials should be made which would include a greater variety of known good submerged cultures. The results of such tests are graphically presented in figure 2. The yields shown, in all cases, represent the average maxima for triplicate shaken-flask cultures. Stock cultures were employed for these tests, but in the case of NRRL 1984.A a reisolation from an earlier shaken-flask culture was also included. The total yields obtained in this experiment with some strains, notably NRRL 1951.B25 and Stanford 25099 and 35217, were below the levels usually produced by these strains when grown under similar culture conditions. This, however, was not believed to invalidate the usefulness of the results as a measure of the relative capacities of the different cultures to produce chloroform-insoluble penicillin.

Confirming the results obtained by Ward and associates in larger laboratory apparatus, best yields of chloroform-insoluble penicillin were obtained from NRRL 1984.A. Total yields from the parent culture, NRRL 1984, were appreciably lower, but the proportion of penicillin X was essentially the same as in the more productive substrain. The same general relationship applied to Minn. 9SS251, a substrain of Minn. R-13 (= NRRL 1984) of monospore origin distributed by Professor Christensen. Substantially higher total yields were

obtained with X-1612 than with any other culture, but yields of chloroform-insoluble penicillin were very low. Low yields were likewise obtained with NRRL 1951.B25, the parent of X-1612, and Stanford strains 25099 and 34217, likewise derived from NRRL 1951.B25. Yields of penicillin X from strains

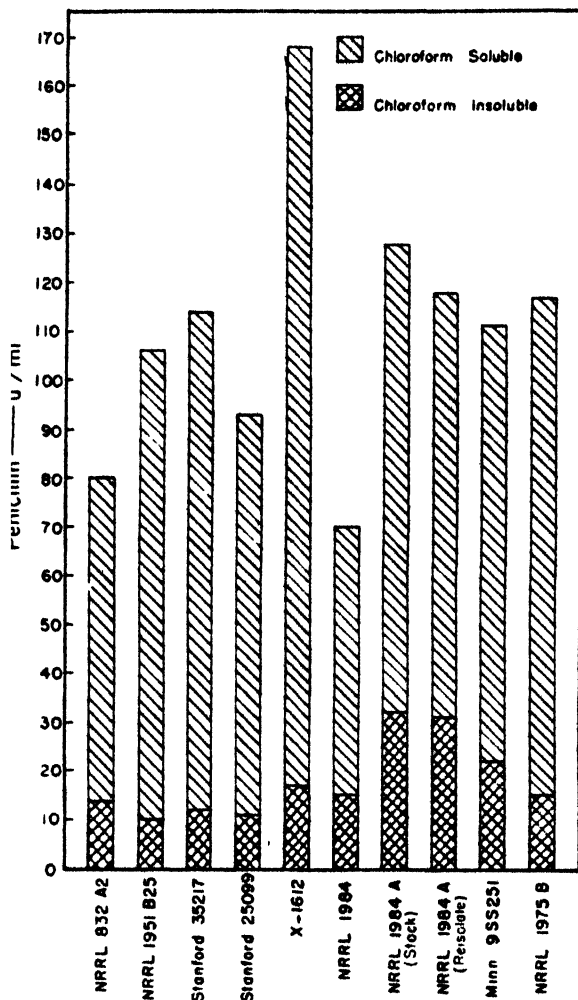


FIG. 2. COMPARATIVE YIELDS OF TOTAL PENICILLIN AND OF CHLOROFORM-INSOLUBLE PENICILLIN, OR PENICILLIN X, PRODUCED BY NINE SELECTED "SUBMERGED" CULTURES
Tested in shaken flasks.

NRRL 832.A2 and 1975.B were intermediate between those obtained from 1984.A and the much lower yields of 1951.B25 and derivative strains.

Upon the basis of these tests, it appeared that cultures of common ancestry tended to show similarities in their ability to produce penicillin X. Further efforts, therefore, were directed toward the development of improved substrains from the most promising culture available, NRRL 1984.A.

Selection of natural variants of NRRL 1984.A. Applying the general methods previously employed for the isolation of such superior penicillin-producing strains as NRRL 1249.B21 and 1951.B25 (Raper and Alexander, 1945), the writers attempted to develop naturally occurring variants characterized by the production of substantially increased amounts of penicillin X. Streak plates were made from high-yielding shaken-flask cultures and observed for the development of obvious cultural variants differing from normal in such characteristics as color, colony texture, rate of growth, and the amount of sporulation. Altogether about 50 substrains were selected and subsequently tested in shaken-flask culture. The majority of these substrains produced yields approximately equal to the parent strain; others, including most of the more striking variants, produced consistently lower yields either of total penicillin, of penicillin X, or of both. A few substrains appeared to be better producers when first tested but failed to show consistently higher yields upon repeated cultivation. Thus the primary objective of these experiments was not realized. They were significant, however, since they demonstrated that NRRL 1984.A, like NRRL 1249.B21, NRRL 1951.B25, and other good penicillin-producing strains, is characterized by great cultural variability.

Ultraviolet irradiations. Our attention was next directed toward the use of ultraviolet radiation as a possible means of developing substrains of 1984.A capable of producing increased amounts of penicillin X. The technique had been used successfully in the development of better itaconic-acid-producing strains of *Aspergillus terreus* (Hollaender, Raper, and Coghill, 1945; Raper, Coghill, and Hollaender, 1945; Lockwood, Raper, Moyer, and Coghill, 1945), and at Stanford University Professor G. W. Beadle and associates had employed it with some success in developing molds characterized by increased penicillin production. Working with other fungi, primarily species of *Neurospora*, and employing both X-ray and ultraviolet radiation, the Stanford group (Beadle and Tatum, 1941; Bonner, Tatum, and Beadle, 1943; Horowitz and Beadle, 1943; Tatum and Beadle, 1942a, 1942b) had developed a whole series of mutations possessing altered biochemical properties. By means of X-ray radiation, Dr. M. Demerec and associates had produced a mutant, X-1612, characterized by greatly increased penicillin production. The technique of radiation as a suitable means of artificially producing mold mutations was thus well established, and it seemed reasonable to believe that mutations might be produced in which the ratio of different penicillins would be altered sufficiently to produce a substantially greater proportion of penicillin X.

First irradiation of NRRL 1984.A. Conidia from a week-old tube culture (25 by 150 mm) of 1984.A growing upon sporulation media (Moyer and Coghill, 1946a) were thoroughly suspended in sterile water and irradiated in the manner already described. The suspension contained approximately 250,000,000 conidia per ml, and, in this case, the lamp was supported at a distance of 2 inches above the liquid surface. Samples were removed and plated as noted above, and the resulting plates were incubated at room temperature. The plates were examined and population counts made after 4 days. The intensity of the irradi-

ation was misjudged, with the result that the killing curve was unusually steep. Less than 1 per cent of the conidia remained viable after 2 minutes' exposure, and only occasional colonies developed in the 4-minute-exposure plates. Isolations upon agar slant tubes were made at random from the 2-minute plates, a total of 50 colonies being picked off in this initial series. These were subsequently replanted upon sporulation media to insure the development of adequate

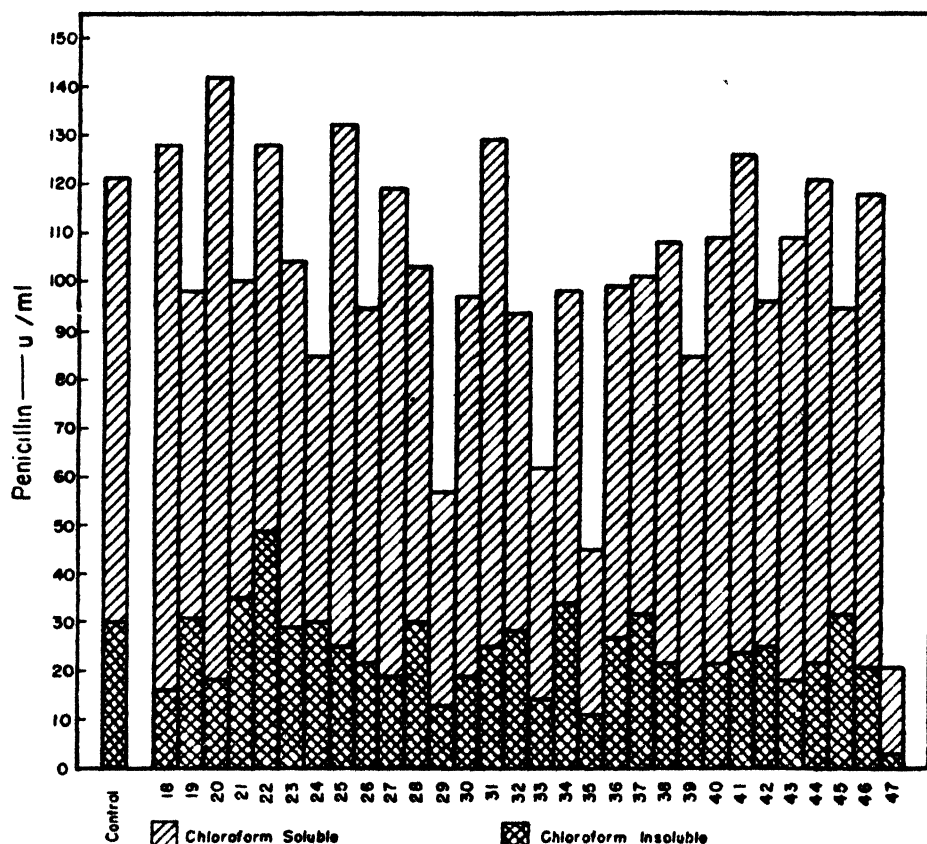


FIG. 3. COMPARATIVE YIELDS OF TOTAL PENICILLIN AND OF CHLOROFORM-INSOLUBLE PENICILLIN, OR PENICILLIN X, PRODUCED BY NRRL 1984.A (CONTROL) AND THIRTY SUBSTRAINS OF 1984.A RESULTING FROM CONIDIA IRRADIATED WITH ULTRAVIOLET

Tested in shaken flasks.

conidia for seeding the test production flasks. Limitations in the number of assays that could be assigned to this work necessitated the testing of these cultures during two different weeks, but the results obtained in the two experiments are believed to be wholly comparable. Average total yields and average yields of chloroform-insoluble penicillin for a block of 30 consecutively numbered cultures that are representative of the whole series are presented in figure 3. The control represents the average of yields produced by four unirradiated stocks.

The cultures resulting from irradiated conidia showed wide variation both in total penicillin production and in the formation of chloroform-insoluble penicillin, or penicillin X. Although they could not be so classified in all cases, these cultures tended to fall into four more or less tangible groups: (1) cultures producing low total yields but with penicillin X in approximately normal proportion to the whole; (2) cultures producing high total yields but relatively small amounts of penicillin X; (3) cultures producing approximately normal yields of both total penicillin and of penicillin X (these were believed to represent, in the main, cultures resulting from conidia only slightly or not at all affected by the radiation); and (4) a single culture, no. 22, producing a normal total yield and an unusually high ratio of penicillin X. The latter culture was of immediate and particular interest, and in the next succeeding experiment was retested with no. 18 (high total but low penicillin X) and no. 25 (high total and intermediate penicillin X) to determine whether the observed ratios were reproducible. The results were wholly confirmatory; no. 18 showed total yields averaging 115 units per ml, of which penicillin X represented 20 units per ml; no. 25 showed total yields averaging 124 units per ml, of which penicillin X represented 30 units per ml; whereas no. 22 showed total yields averaging 155 units per ml, of which penicillin X represented 54 units per ml.

Two other cultures, not included in figure 3, were of special interest: no. 11 was characterized by tan-colored rather than blue-green conidia, but produced normal amounts of total penicillin and of penicillin X; no. 16 was characterized by restricted, light sporulating colonies and when tested produced no detectable quantity of penicillin.

Second irradiation of NRRL 1984.A. In the hope of obtaining an even better penicillin X producer than no. 22 of the first series, conidia of NRRL 1984.A were again irradiated. In this case, the lamp was placed at a distance of 4 inches above the surface of the suspension, and the killing rate was much less rapid. Fifty random isolations were made from the 4-, 6-, and 8-minute-exposure plates, recultivated upon sporulation media, and tested for their capacity to produce penicillin. In general, the results paralleled those obtained from the first series of irradiations, but no new culture was found which equaled no. 22 of the first series in the proportion of chloroform-insoluble penicillin produced.

Irradiation of NRRL 1984.N22. For the third and last series, it was decided to irradiate conidia of culture no. 22, or NRRL 1984.N22 as it was now designated. The procedure employed was the same as that used for the second irradiation. The killing rate was regarded as satisfactory, and random isolations were made from the 1-, 4-, 6-, and 8-minute-exposure plates. Altogether 80 cultures resulting from "twice" irradiated conidia were surveyed for penicillin production in three successive experiments. The results obtained from 30 consecutively numbered cultures, all from conidia exposed for 4 minutes, are graphically presented in figure 4. The general groupings noted for the first series as shown in figure 3 could be applied here, although only one culture, no. 30, showed a reasonably high total yield coupled with a low penicillin X content and no strain showed an outstanding increase in the proportion of penicillin X

produced. In the initial test, cultures no. 14 and no. 29 appeared most promising, both from the standpoint of total production and penicillin X formation. But when retested, no. 14 produced average total yields of 125 units per ml, of which 68 units per ml represented penicillin X; and no. 29 produced average

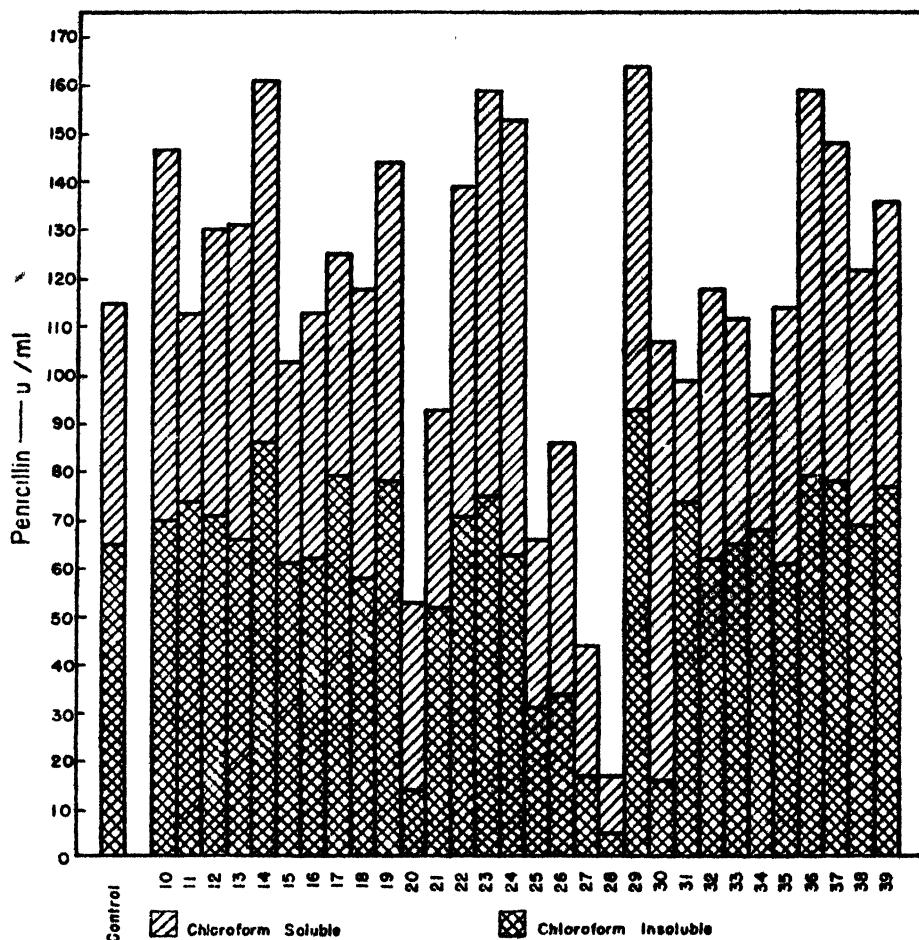


FIG. 4. COMPARATIVE YIELDS OF TOTAL PENICILLIN AND OF CHLOROFORM-INSOLUBLE PENICILLIN, OR PENICILLIN X, PRODUCED BY NRRL 1984.N22 (CONTROL) AND THIRTY SUBSTRAINS OF 1984.N22 RESULTING FROM CONIDIA IRRADIATED WITH ULTRAVIOLET.

Tested in shaken flasks.

total yields of 124, of which 61 units per ml represented penicillin X; whereas in the same experiment the parent culture 1984.N22 produced an average total yield of 115 units per ml, of which 65 units per ml represented penicillin X. Among the cultures of this irradiation series which are not included in figure 4, there were two others which gave high yields of total penicillin and of penicillin X; but like

nos. 14 and 29, these failed to show any apparent improvement in the amount of either total penicillin or penicillin X production when retested. In none of these cases were the observed differences regarded as significant.

The 80 cultures resulting from "twice" irradiated conidia included a number of striking cultural mutations which were of considerable interest from a mycological point of view. To some degree, the yields of penicillin produced by these could be correlated with particular cultural types. Most abundant among the cultures were forms producing colonies essentially normal in appearance, and these generally produced good total yields of penicillin, of which approximately half represented penicillin X. It can be assumed that many of these developed from conidia not affected by the radiation. Two cultures were characterized by white to light buff conidia, and from one of these fair yields were obtained, from the other good yields. A few cultures produced restrictedly growing but heavily sporulating colonies, and these gave uniformly good yields. Another group of cultures characterized by restrictedly growing, but close-textured and lightly sporulating, colonies produced poor yields. A single culture that grew very sparingly upon Czapek's solution agar and that appeared to possess some nutritional deficiency grew well in shaken-flask cultures and produced high total yields of penicillin and of penicillin X.

It is to be regretted that the writers did not have sufficient time and facilities at their disposal, during the course of this investigation, to make a more thorough mycological study of the interesting cultural mutations which were produced, or to survey a sufficiently large number of irradiated substrains to permit some statistical analyses.

In view of the limited number of cultures studied, the question arises as to whether NRRL 1984.N22 represents an induced mutation, or whether it represents merely a natural variant, presumably of monospore origin, which was fortuitously isolated from the dilution plates. No proof can be presented on this matter, but we do know that among approximately 50 substrains, representing obvious variants and other selected isolates of natural origin, none appeared appreciably better than the parent 1984.A. Furthermore, in two other irradiation series no substrain appeared which showed consistently higher yields than the parent stock under investigation. It is, therefore, presumed that the high penicillin-X-producing strain, NRRL 1984.N22, represents a true mutation of NRRL 1984.A, artificially induced by ultraviolet radiation. The performances of both parent and mutant substrain are consistent and would seem to support this view. The latter regularly produced yields of penicillin X amounting to about 50 per cent of the total, as measured by differential assay, which is about 65 to 70 per cent of the total yields upon a weight basis.

Comparison of NRRL 1984.A and 1984.N22. It should be borne in mind that a considerable degree of variation in yields of penicillin can always be expected in shaken-flask tests from one experiment to another (Raper and Alexander, 1945), and that it is often necessary to test a culture repeatedly to be sure of its improved performance. Such evidence has been provided in ample measure for

NRRL 1984.N22. In at least a dozen experiments in which this strain has been grown in shaken-flask culture as the principal object of investigation, or as the control for additional irradiated cultures, it has produced total yields of penicillin ranging from 100 to 130 units per ml, of which amount an average of approximately one-half of the activity represented chloroform-insoluble penicillin, or penicillin X.

The relative productivity of the two strains is clearly demonstrated in table 1 in which are presented representative yields from a series of different experiments. The assay values presented are averages for duplicate or triplicate flasks and represent maximum yields, usually occurring on the fifth day. Assay ratios for

TABLE 1

Penicillin production by Penicillium chrysogenum NRRL 1984.A and NRRL 1984.N22, an ultraviolet-induced mutation characterized by substantially increased penicillin X production, in shaken-flask cultures

NRRL 1984.A						NRRL 1984.N22					
Expt. no.	Differential assay					Expt. no.	Differential assay				
	Whole broth	Chloroform- extracted broth		Assay ratios			Whole broth	Chloroform- extracted broth		Assay ratios	
	B-313	B-558(R)	B-313	On ex- tracted broth	On pure pen. X		B-313	B-558(R)	B-313	On ex- tracted broth	On pure pen. X
	u/ml	u/ml	u/ml				u/ml	u/ml	u/ml		
155	127	49	28	1.7	1.5	160	128	96	49	1.9	1.7
156	110	58	27	2.1	1.8	161	97	77	54	1.4	1.4
157	128	47	32	1.5	1.6	164	117	115	54	2.1	1.9
157	118	44	31	1.4	1.6	165	97	106	60	1.8	1.8
159	111	52	31	1.7	2.0	165	96	116	55	2.1	1.8
165	96	46	28	1.6	1.8						
166	107	40	25	1.6	1.7	170	115	81	64	1.3	1.4
167	119	61	29	2.1	2.0	171	113	108	61	1.8	1.8
170	121	32	26	1.3	1.3	172	115	103	65	1.6	1.7

the chloroform extracts have been calculated and are presented parallel with assay ratios for pure penicillin X as determined on the same days.

Culturally the mutant NRRL 1984.N22 cannot be distinguished from the parent NRRL 1984.A. Both are characterized by heavily sporulating, essentially velvety colonies which fit reasonably well the species concept for *Penicillium chrysogenum* Thom.

Vat fermentations and the recovery of penicillin X. The capacity of NRRL 1984.N22 to produce penicillin X in the proportions indicated above was verified by conducting 600-liter fermentations in a pilot plant vat fermenter, and by subsequently recovering the penicillin from the fermented culture broth. Two of these fermentations were made by R. G. Benedict and D. H. Trautner. In the second of these runs the operational details and the yields obtained were as follows:

Medium: Lactose 2.5 per cent, corn steep liquor 6 per cent (by vol.), calcium carbonate 0.5 per cent, and tap water to make 600 liters.

Conditions: Temperature—24 to 25C
Aeration—600 liters per minute
Agitation—250 rpm

Inoculum: 12 liters of germinated spores of *P. chrysogenum* NRRL 1984.N22, age 2 days.

Samples were removed at appropriate intervals throughout the period of the fermentation and assayed for total penicillin and penicillin X. At 90 hours, approximately 50 per cent of the total penicillin assayed as chloroform-insoluble penicillin, or penicillin X. By weight this represented approximately 67 per cent of the total penicillin.

Assay values at 90 hours (units/ml)

WHOLE BROTH		BROTH AFTER CHLOROFORM EXTRACTION		ASSAY RATIO ON PURE PEN. X
<i>S. aureus</i> , NRRL B-313	<i>B. subtilis</i> , NRRL B-558(R)	<i>S. aureus</i> , NRRL B-313	<i>B. subtilis</i> , NRRL B-558(R)	
u/ml	u/ml	u/ml	u/ml	
192	273	102	136	1.4

The fermentation was terminated at 90 hours, and the recovery operations were taken over by J. L. Wachtel. From 200 liters of filtered culture liquor, more than 2 grams of crystalline sodium penicillin X were isolated in two crops of 1,820 and 390 mg, respectively.⁵ Both crops gave X-ray diffraction patterns characteristic of penicillin X. The first crop assayed 920 units per mg against *S. aureus*, B-313, and its assay value against *B. subtilis*, B-558(R), was 1.6 times greater. The ultraviolet absorption was correct for sodium penicillin X, and carbon and hydrogen values on the first crop were in good agreement with theory. In concluding his report of this work, Dr. Wachtel states: "This isolation experiment shows without doubt that the chloroform-insoluble penicillin produced by *P. chrysogenum* NRRL 1984.N22 is penicillin X, approximately 50 per cent of the activity being due to this analogue."

SUMMARY

Of several good penicillin-producing strains investigated, *Penicillium chrysogenum* NRRL 1984.A, a substrain derived from Minn. R-13, was found to be the

⁵ In industrial and laboratory practice a recovery of penicillin of "commercial grade" representing 50 per cent of the potency in the original broth is considered satisfactory. The product thus obtained usually contains 50 to 75 per cent penicillin by weight, with the remainder representing impurities. Additional losses are encountered in the successive steps leading to the production of a crystalline salt from this material. The isolation of 2.21 g of pure sodium penicillin X from a potential of roughly 20 g in the whole broth (as measured by differential assay) is considered satisfactory.

best producer of chloroform-insoluble penicillin, or penicillin X, when grown in submerged culture.

By ultraviolet irradiation, a substrain of 1984.A, designated NRRL 1984.N22, was developed, which produced substantially higher yields of penicillin X, generally amounting to approximately 50 per cent of the total yield as measured by differential assays, and representing approximately 65 to 70 per cent of the total yield upon a weight basis.

The superior performance of NRRL 1984.N22 as a producer of penicillin X was repeatedly observed in shaken-flask cultures, and was subsequently demonstrated in 600-liter vat fermentations. From one of these fermentations pure penicillin X was isolated in a quantity which verified the approximate correctness of the survey data based upon differential assays only.

The technique of irradiation, the method of performing chloroform extractions, and the theory and practice of the differential assay used in demonstrating the presence and determining the amount of penicillin X are presented.

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THE INFLUENCE OF CHANGES IN CONCENTRATION OF SODIUM HYDROXIDE UPON ITS BACTERICIDAL ACTIVITY

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Although the literature contains a number of reports regarding the bactericidal efficiency of certain specified concentrations of sodium hydroxide against various test organisms, very little of this information is presented in a manner suitable for mathematical analysis. To obtain the data required for analysis, an experimental study was made of the bactericidal efficiency of sodium hydroxide. This study was conducted along the lines of a previously described study of the bactericidal efficiencies of some of the phenols and alcohols (Tilley, 1939).

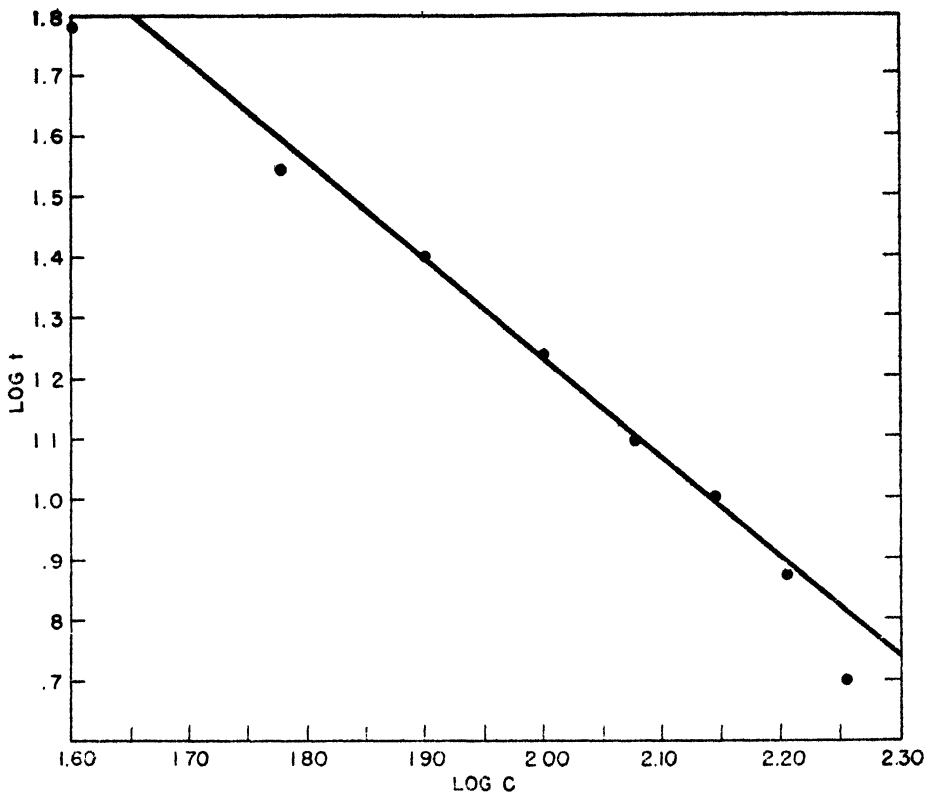
EXPERIMENTAL PROCEDURE

Bactericidal efficiency was determined by the Rideal-Walker technique, modified as follows: Either infusion broth or FDA beef extract broth was used instead of R-W standard broth; instead of a standardized dropping pipette a 1-ml pipette graduated into tenths was used to measure out the 0.5 ml of culture required for the test; medication temperatures were sometimes 20 C and sometimes 30 C instead of 15 C to 18 C; dilutions were made in sterile Erlenmeyer flasks instead of in cylinders; fixed amounts of the stock solution of disinfectant were added to varying amounts of distilled water, all measured with sterile standardized pipettes; the time of exposure was extended beyond 15 minutes, subcultures being made at 2.5-minute intervals when exposure varied from 2.5 to 30 minutes, at 5-minute intervals when exposures varied from 30 to 80 minutes, and at 10-minute intervals when exposures were beyond 80 minutes; and *Staphylococcus aureus* and *Escherichia coli* were used as test organisms instead of *Eberthella typhosa*.

The bactericidal efficiency of sodium hydroxide was determined by the technique described above, and from the experimental data thus obtained values of the concentration exponent, n , were calculated as described in a previous paper (Tilley, 1939) by the equation, $n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$ etc., for results presented in tabular form, and by the equation, $n = \frac{y_2 - y_1}{x_1 - x_2}$ for results presented graphically.

EXPERIMENTAL AND CALCULATED RESULTS

Experimental results obtained with *Staphylococcus aureus* as the test organism are shown in table 1 and graph 1. When calculations are based on results with concentrations between 40 and 160 g in 10,000 ml, the value of the concentration exponent, n , is 1.58 when calculated from results shown in table 1, and 1.62 when calculated from results shown in graph 1. The result obtained



GRAPH 1. BACTERICIDAL EFFICIENCY OF SODIUM HYDROXIDE AGAINST STAPHYLOCOCCUS AUREUS, $n = 1.62$

TABLE 1

Bactericidal efficiency of sodium hydroxide against Staphylococcus aureus in ordinary beef infusion broth

C G IN 10,000 ML.	t KILLING TIME IN MINUTES	LOG C	LOG t	LOG C_1 — LOG C_2 ETC.	LOG t_1 — LOG t_2 ETC.	n
180	5	2.25527	0.69897			
160	7.5	2.20412	0.87506	5115	17609	3.44*
140	10	2.14613	1.00000	5799	12494	2.15
120	12.5	2.07918	1.09691	6695	09691	1.44
100	17.5	2.00000	1.24304	7918	14613	1.84
80	25	1.90309	1.39794	9691	15490	1.59
60	35	1.77815	1.54407	12494	14613	1.17
40	60	1.60206	1.77815	17609	23408	1.33
Average.....						1.58

Experiment conducted at 20 C.

* Anomalous and therefore omitted in calculating average.

TABLE 2

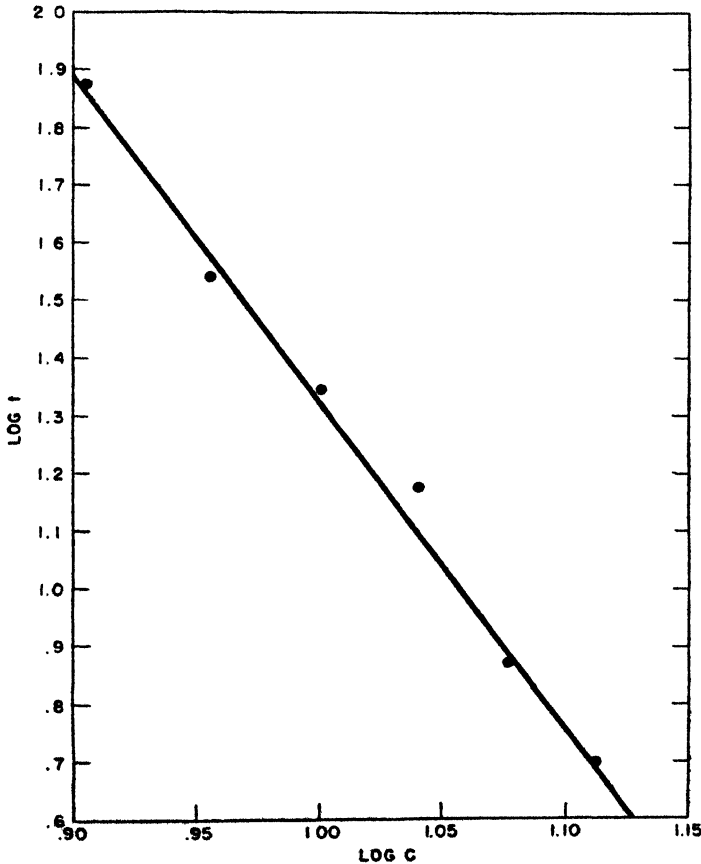
Bactericidal efficiency of sodium hydroxide against Escherichia coli grown in ordinary beef infusion broth

C G IN 10,000 ML	t KILLING TIME IN MINUTES	LOG C	LOG t	LOG $C_1 -$ LOG C_2 , ETC.	LOG $t_1 -$ LOG t_2 , ETC.	n	LOG A
13	5.0	1.11394	0.69897				6.93
12	7.5	1.07918	0.87506	3476	17609	5.00	6.92
11	15.0	1.04139	1.17609	3779	30103	7.96	7.00
10	22.5	1.00000	1.35218	4139	17609	4.25	6.95
9	35.0	0.95424	1.54407	4576	19189	4.19	6.89
8	75.0	0.90309	1.87506	5115	33099	6.47	6.93
7	*	0.84510					
6	†	0.77815					
Average.....						5.6	6.93

Experiment conducted at 30 C.

* Calculated disinfection time was 159 minutes. Observed time was between 24 and 48 hours.

† Calculated disinfection time was 372 minutes. Growth occurred in subcultures after 7 days' exposure.



GRAPH 2. BACTERICIDAL EFFICIENCY OF SODIUM HYDROXIDE AGAINST ESCHERICHIA COLI,
 $n = 5.6$

with a concentration of 180 g in 10,000 ml was anomalous and was not used in calculating this value. With this exception, the results shown indicate that, within the concentration range employed, the process of disinfection follows an approximately logarithmic course.

TABLE 3

Bactericidal efficiency of sodium hydroxide against Escherichia coli grown in beef extract broth

C g IN 10,000 ML	t KILLING TIME IN MINUTES	LOG C	LOG t	LOG C ₁ - LOG C ₂ , ETC.	LOG h ₁ - LOG h ₂ , ETC.	n
10	5	1.00000	0.69897			
9	7.5	0.95424	0.87506	4576	17609	3.84
8	10	0.90309	1.00000	5115	12494	2.44
7	17.5	0.84510	1.24304	5799	24304	4.19
6	27.5	0.77815	1.43933	6695	19629	2.98
5	60	0.69897	1.77815	7918	33882	4.29
Average.....						3.54

Experiment conducted at 30 C.

TABLE 4

Bactericidal efficiency of sodium hydroxide against Escherichia coli grown in special beef infusion broth

C g IN 10,000 ML	t KILLING TIME IN MINUTES	LOG C	LOG t	LOG C ₁ - LOG C ₂ , ETC.	LOG h ₁ - LOG h ₂ , ETC.	n	LOG A
14	5	1.14613	0.69897				7.72
13	7.5	1.11394	0.87506	3219	17609	5.47	7.70
12	12.5	1.07918	1.09691	3476	22185	6.38	7.71
11	20	1.04139	1.30103	3779	20412	5.40	7.68
10	35	1.00000	1.54407	4139	24304	5.87	7.67
9	75	0.95424	1.87506	4576	33099	7.23	7.72
8	180	0.90309	2.20412	5115	32906	6.43	7.74
7	*	0.84510					
6	†	0.77815					
Average.....						6.13	7.71

Experiment conducted at 30 C.

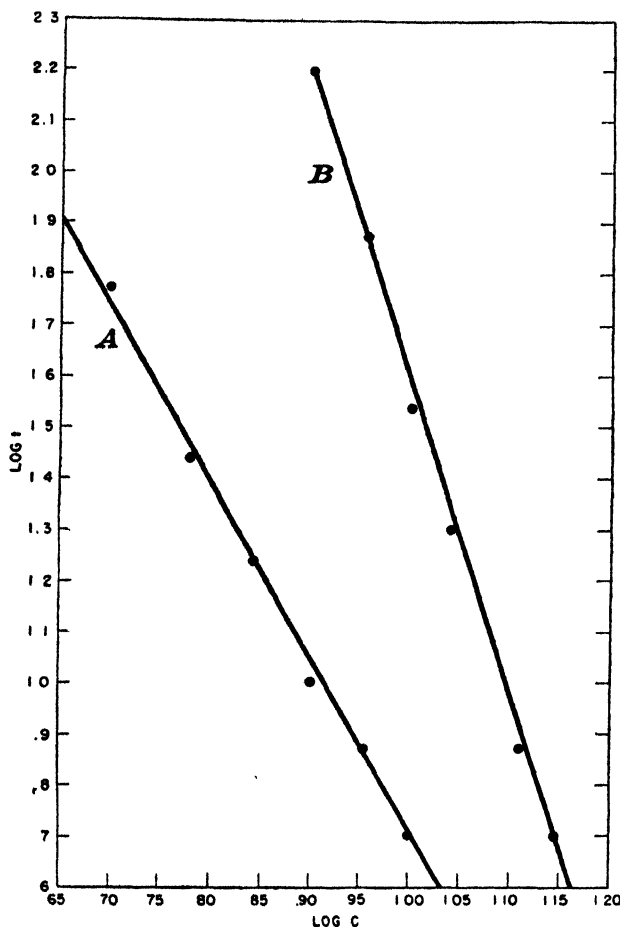
* Time calculated by equation, $\log t = \log A - n \log C$ with the values of n and $\log A = 339$ minutes. Observed time was between 24 and 48 hours.

† Time calculated as above was 871 minutes. Growth occurred in subcultures after 7 days' exposure.

Similar experimental results with *Escherichia coli* as the test organism are shown in table 2 and graph 2. When calculations are based on concentrations between 13 and 8 g in 10,000 ml, the value obtained for n is 5.6, and the process of disinfection appears to follow an approximately logarithmic course. However, it will be noted in table 2 that there is a sharp break between a concentration

of 8 g in 10,000 ml and a concentration of 7 g in 10,000 ml. The calculated disinfection times shown in the table were obtained by using the values $n = 5.6$ and $\log A = 6.93$ in the equation $\log t = \log A - n \log C$.

Tables 3 and 4 present the results of experiments with cultures of *Escherichia coli* grown in FDA beef extract broth and in a special beef infusion broth contain-



GRAPH 3. BACTERICIDAL EFFICIENCY OF SODIUM HYDROXIDE AGAINST *ESCHERICHIA COLI*

A. Beef extract broth. $n = 3.5$

B. Special beef infusion broth. $n = 6.2$.

ing 2 per cent peptone, respectively. The results with both media are also shown in graph 3. It is evident that even in the concentration ranges where the process of disinfection follows an approximately logarithmic course, the rate of disinfection is markedly affected by the amount of organic matter present. Likewise a comparison of results in tables 2 and 4 shows that a comparatively small increase in organic matter causes an appreciable increase in the disinfection

time. The results shown in these tables also indicate that the effective concentration range is sharply limited at 6 g per 10,000 ml.

Data presented in table 5 show the effect of varying amounts of organic matter upon the pH of sodium hydroxide solutions and upon their bactericidal efficiency against *Escherichia coli*. The pH values of the original solutions were determined with the Beckman pH meter, using a glass electrode especially designed for use in solutions containing high alkali concentrations. The pH values of the test mixtures were usually determined with the pH meter after standing 24 hours. When growth was noted in subcultures from test mixtures, the pH values of these mixtures were determined colorimetrically, using the standard

TABLE 5

Effect of varying amounts of organic matter upon the pH and upon the bactericidal efficiency of sodium hydroxide solutions against Escherichia coli

NaOH G IN 10,000 ML	ORIGINAL PH VALUES	A		B		C	
		Final pH values	Killing time in minutes	Final pH values	Killing time in minutes	Final pH values	Killing time in minutes
14	12.50	11.7	5.0	10.5	60		
12	12.45	11.4	12.5	10.4	100		
11	12.40	11.1	20.0	10.3	*		
10	12.35	11.0	35.0	10.2	*	11.85	5.0
9	12.30	10.6	75.0	10.1	*	11.75	7.5
8	12.25	10.3	160.0	9.0	†	11.55	10.0
7	12.15	9.7	†			11.30	17.5
6	12.10	9.5	‡			11.00	27.5
5	12.00					10.50	60.0

Experiment conducted at 30 C.

A and B test cultures grown in special beef infusion broth. A—no additional organic matter. B—20 per cent serum added. C—test cultures grown in beef extract broth; no serum added.

* Growth after 3 but not after 24 hours.

† Growth after 24 but not after 48 hours.

‡ Growth after 7 days.

solutions of Kolthoff and Vlesschouwer as described by Clark (1928). When these standard solutions were freshly prepared, there was good agreement between the results obtained with them and the results obtained with the glass electrode.

The results shown in table 5 indicate that with *E. coli* as the test organism the original concentrations and pH values of sodium hydroxide solutions are not significant. It is the final pH of the test mixtures that should be taken into consideration. These results, as well as the results of other unreported experiments, indicate that when the final pH falls below 10, bactericidal activity disappears. The results obtained with *Staphylococcus aureus* indicate that this statement is also true for that organism.

SUMMARY

The bactericidal efficiency of sodium hydroxide against *Staphylococcus aureus* and *Escherichia coli* was determined by a modified Rideal-Walker technique. From the resulting experimental data, values for the concentration exponent n were calculated by the formula, $n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$ for results presented in tabular form, and by the formula, $n = \frac{y_2 - y_1}{x_1 - x_2}$ for results presented in graphical form.

When test cultures were grown in ordinary beef infusion broth, the values obtained for n were approximately 1.6 for *Staphylococcus aureus* and 5.6 for *Escherichia coli*.

Within a comparatively limited range of concentrations the bactericidal action of sodium hydroxide against both test organisms followed, with either small or large amounts of organic matter, an approximately logarithmic course. However, the slope of the curve was related to the organic matter present. Bactericidal efficiency was correlated with the final pH of the test mixtures. When the pH fell below 10, efficiency suddenly dropped, and at a pH between 10 and 9 disappeared altogether.

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AN IMPROVED METHOD FOR DIFFERENTIATING ACID-FORMING FROM NON-ACID-FORMING BACTERIA

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Excessive acid formation in some biological processes may be undesirable because of enzyme inhibition and the production of undesirable by-products. For instance, in the alcoholic fermentation by yeast, the presence of sufficient acid-forming organisms will lower the pH enough to inhibit or destroy α -amylase. Bacteria gain entrance to the fermenters through the equipment, water, and malt. Consequently, a yeast fermentation of grain is always contaminated with a greater or lesser number of bacteria. The method to be described enables one to obtain the ratio of acid-forming to non-acid-forming bacteria in such a fermentation. The method could be readily applied to other circumstances in which this problem is involved.

The medium used, which is a modification of the one used by Garey *et al.* (1945), consists of:

Tomato juice (filtered).....	300 ml
Yeast extract.....	5 g
Glucose.....	5 g
Salts A*.....	5 ml
Agar.....	15 g
Distilled water to.....	1,000 ml

Neutralize to pH 7.0 with NaOH

* 25 g each of monobasic and dibasic potassium phosphate per 250 ml of distilled water.

This medium is sufficiently light in color to make counting of colonies easy by the tube method (Garey *et al.* 1945). The medium is dispensed in 100-ml amounts in 125-ml Erlenmeyer flasks; 0.4 per cent CaCO_3 is added with 0.2 ml of a 1.6 per cent alcoholic solution of bromcresol purple to 100 ml of medium. If desired, the indicator may be added after sterilization. It was found expedient to add the CaCO_3 from a water suspension by use of a pipette. This medium, when used for plate counts, is satisfactory for determining the ratio of acid-forming to non-acid-forming bacteria. After the plates are poured, they should be cooled rapidly on a flat, cold surface to keep the CaCO_3 in an even suspension through the medium. The plates are incubated at 37 C for 48 hours.

The CaCO_3 prevents the diffusion through the agar of the acid formed by acid-forming colonies. As a result, the indicator changes color only in a small zone surrounding the colony. Without the CaCO_3 , acid from strong acid-forming colonies will diffuse over the entire plate making it impossible to distinguish between acid-forming and non-acid-forming colonies.

Bacterial colonies on this medium appear in four types: (1) Strong acid-producing colonies, which change the indicator from purple to yellow and dissolve the CaCO_3 , resulting in the formation of clear zones around the colonies. (2) Mild acid-forming colonies, which change the color of the indicator from purple to yellow but form no clear zones. (3) Neutral colonies which cause no change in the agar. (4) Alkali-forming colonies, which give the agar a deeper purple color. The appearance of pure cultures of various bacteria is described in table 1.

TABLE 1

Appearance of colonies of known bacteria on medium containing CaCO_3 and bromcresol purple

CULTURE	APPEARANCE
<i>Bacillus subtilis</i>	Slight alkaline reaction around the colonies
<i>Escherichia coli</i>	Slight yellow cloudy zone surrounds the colony
<i>Serratia marcescens</i>	Yellow cloudy zone surrounds the colony
<i>Staphylococcus aureus</i>	Very slight yellowing of the agar
<i>Streptococcus faecalis</i>	Clear yellow areas about 2 mm in diameter surround colonies
Acid-forming organism isolated from distillers' mash	Clear yellow zones about 5 mm in diameter surround colonies

This method has been extensively used during a study of the origin of bacterial contamination in yeast fermentation. The plate counts secured on this special agar agree very well with the counts on the same medium without CaCO_3 or bromcresol purple. They also agree with tube counts as described by Garey *et al.* (1945). More difficulty is encountered when fermenters "set" with yeast are plated. The yeast overgrows the plate and obscures the bacteria, but the total bacterial count can easily be determined by means of a tube count. *Saccharomyces cerevisiae* itself will give a slight yellow color on this agar.

REFERENCE

- GAREY, J. C., RITTSCHOFF, L. A., STONE, L., AND BORUFF, C. S. 1945 A study of cultural methods for the quantitative determination of bacterial populations of distillery mashes. *J. Bact.*, **49**, 307-310.

NOTES

THE PRODUCTION OF STAPHYLOCOCCUS TOXIN IN THE CHORIO-ALLANTOIC FLUID OF THE EMBRYONATED EGG

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By the use of the Wood 46 strain of staphylococcus, toxin showing hemolytic, dermonecrotic, and lethal properties has been produced in embryonated eggs. The chorio-allantoic sac of 12-day chick embryos was inoculated with 0.1 ml of a 10^{-3} dilution of an 18-hour broth culture of the staphylococcus. The inoculated embryos were incubated at 37 C for 2 to 3 days. About half the embryos regularly died. The relatively clear allantoic fluid of the surviving embryos was pooled and centrifuged to remove bacterial cells. Preparations could be stored frozen in a dry-ice chamber without loss of activity; at icebox temperatures activity gradually decreased.

Comparisons of toxin produced in the embryo and in semisolid infusion agar under an atmosphere of 80 per cent oxygen and 20 per cent carbon dioxide showed similar toxicities of the two preparations. When relatively small amounts of toxin are desired, as for class demonstration, the allantoic sac method is less laborious and produces equally good toxin, with less special equipment.

The hemolytic property of the toxins was studied by setting up toxin against a 1 per cent suspension of washed, fresh rabbit red blood cells; incubation was in a water bath at 37 C for 1 hour, followed by icebox temperatures overnight. Both the allantoic fluid and semisolid agar preparations showed hemolysis through a dilution of 1:128 at the end of 1 hour, and 1:512 after icebox storage.

Dermonecrotic action was tested in the skin of rabbits by the intradermal injection of increasing toxin dilutions. Necrosis followed injections of 0.1 ml of 1:10 to 1:20 dilutions of both preparations. The lethal effect was demonstrated by the intravenous injection of mice which were 4 to 5 weeks of age. Of the agar-prepared toxin 0.2 to 0.3 ml produced death in 2 to 5 minutes; the allantoic fluid toxin gave similar results in mice injected with 0.1 to 0.4 ml. In all tests normal allantoic fluid gave negative results.

These results compare favorably with figures of other workers (Blair and Hallman: *J. Infectious Diseases*, **72**, 246; Duthie and Wylie: *Brit. J. Exptl. Path.*, **26**, 130; Johlin and Rigdon: *J. Immunol.*, **41**, 233; Smith: *Brit. J. Exptl. Path.*, **18**, 265).

A STUDY OF STREPTOCOCCI ASSOCIATED WITH SUBACUTE BACTERIAL ENDOCARDITIS

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A collection of 113 cultures of streptococci isolated from 100 cases of subacute bacterial endocarditis has been studied in an attempt to classify them according to their physiological and serological characteristics. The results are tabulated in table 1.

The largest group, which consists of 45 cultures, could not be differentiated from *Streptococcus mitis*, the ordinary "greening" streptococcus found in the normal human throat. Twelve cultures possessed identical, or very similar, physiological properties with *Streptococcus bovis*. The identities of *Streptococcus faecalis*, *Streptococcus agalactiae*, and the group G strain were further confirmed

TABLE 1

SPECIES OR VARIETY	NUMBER OF CULTURES
<i>Streptococcus mitis</i>	45
" <i>Streptococcus s.b.e.</i> "	42
<i>Streptococcus bovis</i>	12
<i>Streptococcus faecalis</i>	5
<i>Streptococcus agalactiae</i>	4
Lancefield group G	1
Unclassified	4
Total	113

by serological methods. The rather low incidence of enterococci and the total absence of *Streptococcus salivarius* strains in this collection should be noted.

One group, which comprises approximately one-third of the cultures studied, seems to represent a hitherto unrecognized variety, or species, and is tentatively designated as "*Streptococcus s.b.e.*" This group of streptococci can be recognized easily by serological methods and by virtue of a rather unique pattern of physiological characteristics. These cultures characteristically produce a greening on blood agar, hydrolyze arginine, ferment inulin but not raffinose, and synthesize large quantities of a polysaccharide from sucrose in broth culture. According to preliminary results (Loewe, Plummer, Niven, and Sherman: J. Am. Med. Assoc., 130, 257) cases of subacute bacterial endocarditis caused by *Streptococcus s.b.e.* do not appear to respond so readily to Loewe's penicillin-anticoagulant therapy as do those cases from which other streptococci have been recovered.

THE BIOTIN REQUIREMENTS OF NEISSERIA SICCA

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Studies on the nutritive requirements of the species of *Neisseria* have shown that some strains of *Neisseria intracellularis* (Frantz: J. Bact., **46**, 757) and some strains of *Neisseria gonorrhoeae* (Welton, Stokinger, and Carpenter: Science, **99**, 372) may be grown successfully in liquid synthetic media. These media are of interest in that they consist only of glucose, organic acids, and salts, and they therefore indicate that these organisms do not require any supplementary growth factors. Preliminary experiments showed that these media failed to support the growth of *Neisseria sicca* (V. Lingelsheim) Bergey *et al.*, but that the medium of Landy and Dicken (J. Lab. Clin. Med., **27**, 1086) as modified for the cultivation of *Staphylococcus aureus* (Landy *et al.*: Science, **97**, 265) and without folic acid, supported abundant growth. Experiments were, therefore, performed to determine whether *N. sicca* required any of the other supplementary growth factors that are included in this medium.

Fourteen strains of *N. sicca* were isolated from the nasopharyngeal region of healthy volunteers. Twenty-four-hour nutrient broth cultures were centrifuged, the cells were washed in physiological saline, and were then resuspended in a volume of saline equal to the original volume of the broth culture. One-tenth ml of a 1:10 dilution of this cell suspension was used as the inoculum. The organisms were grown in colorimeter tubes and the degree of growth measured in a Lumetron model 400 G photoelectric colorimeter after 48 hours' incubation.

Four strains of *N. sicca* were tested for growth in the modified Landy and Dicken medium and in media where each of the supplementary growth factors was individually omitted. Maximum growth was obtained in each of these media except when biotin was omitted. Media without biotin gave a minimal growth which reached its maximum in 24 hours, whereas growth continued for 48 to 72 hours when the media contained biotin. Attempts to serially subculture the organism on media lacking biotin failed, but serial subculture was readily accomplished in media containing biotin. Ten additional strains of *N. sicca* were tested in a medium lacking the supplementary factors and in the same medium fortified with biotin. In each case, growth was greatly stimulated by the addition of biotin. A determination of the minimum biotin requirements for 6 strains indicated that maximum growth was obtained with concentrations of 0.05 to 0.1 millimicrogram per ml. Lower concentrations also stimulated growth, but variable results were obtained with the different strains.

After determining the biotin requirement, the hydrolyzed casein was successfully replaced with a mixture of 19 amino acids (the amino acids of casein hydro-

yzate with the exception of hydroxyproline). Several of the strains were then cultured on a series of media, each of the media lacking one of the 19 amino acids. The omission of any one amino acid did not materially affect the growth of these organisms. Additional experiments showed that Welton's medium for *N. gonorrhoeae* would also support the growth of *N. sicca* if biotin were added. Growth, however, was sparse and did not compare with that obtained in the modified Landy and Dicken medium.

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